

University of Alberta

Characterization of the *bldG* locus in *Streptomyces coelicolor*

by

Dawn Renée Daphne Bignell



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

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Dated: August 25, 2003

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of the *bldG* locus in *Streptomyces coelicolor*** submitted by **Dawn Renée Daphne Bignell** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Microbiology and Biotechnology**.

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Abstract

Streptomyces spp. are filamentous soil bacteria renowned for their morphological complexity and for their ability to produce a wide variety of secondary metabolites, of which antibiotics are most notable. A group of genes of *Streptomyces coelicolor*, the *bld* genes, are involved in the regulation of morphological differentiation, and many of these genes, including the *bldG* gene, are also required for antibiotic production. *bldG* and a downstream open reading frame (designated *orf3*) encode proteins showing similarity to anti-anti-sigma factors (e.g. SpoIIAA and RsbV) and anti-sigma factors (e.g. SpoIIAB and RsbW) of *Bacillus subtilis*, respectively. These proteins in *Bacillus* regulate the activity of sporulation- and stress-response-specific sigma factors that are encoded along with the cognate regulatory proteins. However, a sigma factor gene is not present in the vicinity of *bldG* and *orf3* in *S. coelicolor*. An in-frame deletion mutant of *bldG* was constructed that showed the same phenotype as *bldG* point mutants, whereas deletion of the *orf3* gene resulted in a variety of phenotypes with regards to morphological differentiation and antibiotic production, possibly due to the accumulation of secondary site mutations in the target sigma factor gene. *In vitro* phosphorylation assays demonstrated that, as with other anti-anti-sigma factors, BldG is likely regulated by phosphorylation, and a *bldG* mutant strain expressing the non-phosphorylatable BldG protein was unable to differentiate or produce antibiotics, suggesting that phosphorylation of BldG is necessary for proper development in *S. coelicolor*. DNA microarray experiments revealed a number of genes that may be transcribed by a BldG/ORF3-regulated sigma factor, including *afsS*, which encodes a global regulator of antibiotic

production. Potential mechanisms for how BldG and ORF3 might function to regulate morphological and physiological differentiation in *S. coelicolor* are discussed.

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List of Abbreviations

α	Alpha
Δ	Delta or deletion
γ	Gamma
λ	Lambda bacteriophage
Ω	Omega
ϕ	Phi
σ	Sigma factor
A	Adenine or Alanine
Ab	Antibody
Ap ^R	Apramycin resistance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	Base pair
BS3	<i>Bis</i> (sulfosuccinimidyl)suberate
BSA	Bovine serum albumin
C	Cytosine or cysteine
C-	Carboxy
cAMP	Cyclic adenosine monophosphate
CDA	Calcium dependent antibiotic
cDNA	Complementary DNA
Ci	Curie
cpm	Counts per minute
CTP	Cytidine triphosphate
D	Aspartate
Da	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddNTP	Dideoxynucleoside triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E	Glutamate
EDTA	Ethylenediaminetetraacetic acid

EGS	Ethylene glycol <i>bis</i> (succinimidylsuccinate)
EtBr	Ethidium bromide
F	Phenylalanine
fM	Formyl methionine
FPLC	Fast protein liquid chromatography
G	Guanine or glycine
GTP	Guanosine triphosphate
H	Histidine
HEPES	Hydroxyethylpiperazinethansulfonic acid
I	Isoleucine
IP	Immunoprecipitation buffer
IPTG	Isopropyl β -D-thiogalactopyranoside
ISP-4	International <i>Streptomyces</i> medium
K	Lysine
kb	Kilobase
kDa	KiloDalton
kV	KiloVolt
L	Litre(s) or leucine
LB	Luria Bertani medium
M	Molar or methionine
Mb	Megabase
MBP	Maltose binding protein
ml	Millilitre(s)
mmol	Millimole
mRNA	Messenger RNA
MS agar	Mannitol soy flour agar
MW	Molecular weight
N	Asparagine
N-	Amino
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometre
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
P	Phosphorus or proline
P buffer	Protoplast buffer

PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PSI-BLAST	Position-specific iterated basic local alignment search tool
ppGpp	Guanosine tetraphosphate
pmol	Picomole(s)
Q	Glutamine
R	Arginine
R2YE	Sucrose yeast extract medium
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNAse	Ribonuclease
rRNA	Ribosomal RNA
rpm	Revolutions per minute
S	Serine
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
T	Thymine or threonine
TBS-T	Tris-buffered saline tween-20 buffer
T _m	Melting temperature
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	Tetramethyl ethylene diamine
tRNA	Transfer RNA
TSB	Tripticase soy broth
U	Unit (Enzyme activity measurement)
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volt(s) or five or valine
v/v	volume per volume
W	Tryptophan
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Y
YT

Tyrosine
Yeast extract tryptone medium

Chapter 1:
Introduction

1. Introduction

1.1 General Overview

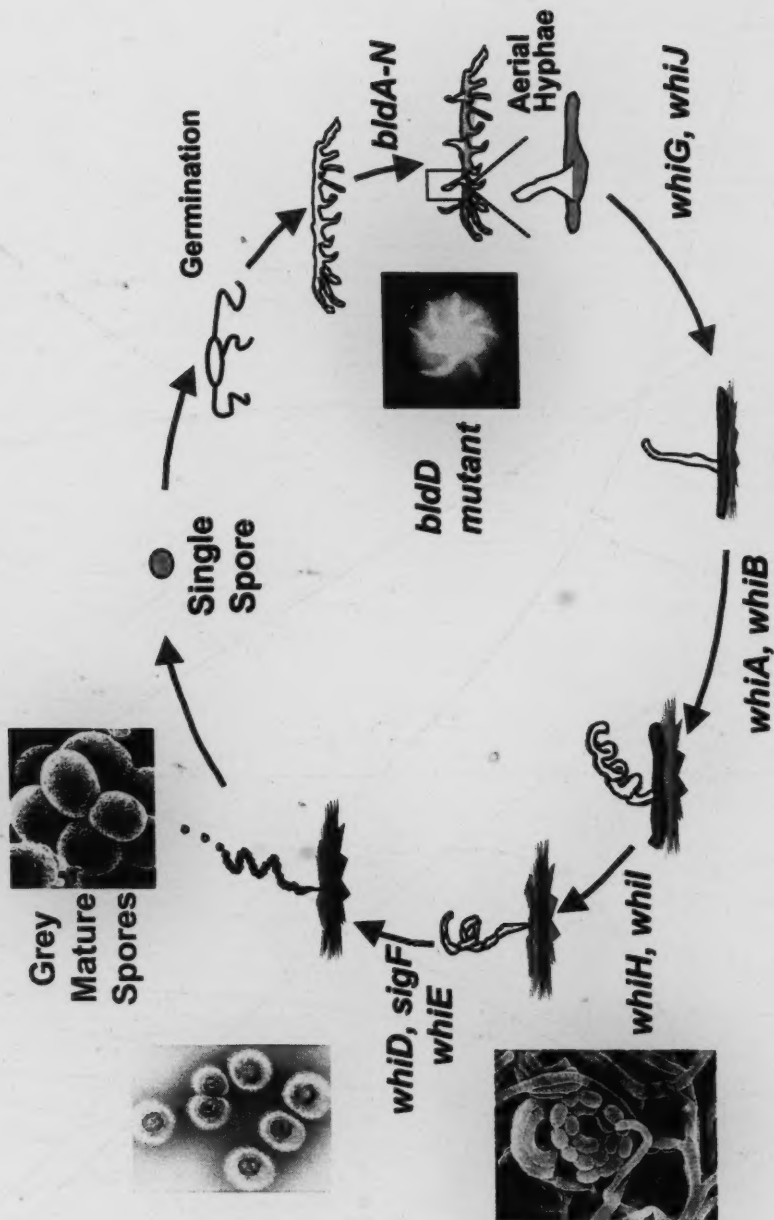
Streptomyces spp. are gram positive, filamentous bacteria that are among the most numerous and ubiquitous organisms found in soil environments (Hodgson, 2000). Organisms of this group play an important role in the recycling of nutrients in the environment due to their ability to produce extracellular hydrolytic enzymes that degrade complex, recalcitrant compounds such as polysaccharides (starch, pectin, chitin), proteins (keratin, elastine) and lignocellulose. Survival in the soil environment is facilitated by the ability to grow as a mat of branched mycelia that can attach to organic particles serving as nutrients, and by the ability to undergo sporulation, a feature that allows for adaptation to variations in physical conditions due to seasonal or weather changes. The spores that are produced are resistant to environmental conditions such as drought, frost, and anaerobic conditions resulting from waterlogged soil, and they also serve as a means of dispersal for an organism that does not display any inherent mobility features (Korn-Wendisch & Kutzner, 1992)

The ability of streptomycetes to compete with other soil microorganisms for nutrients is thought to result in part from their ability to produce a wide variety of secondary metabolites, including a large portion of the naturally-occurring antibiotics that are currently in use (Champness & Chater, 1994). Many of these compounds have applications in medicine as antibacterial, antiviral, antitumor agents and immunosuppressants, while others such as herbicides, insecticides, antifungal and antiparasitic agents are important for agriculture (Korn-Wendisch & Kutzner, 1992).

Interest in these organisms from an industrial standpoint stems mainly from the desire to produce large quantities of antibiotics in a cost-efficient manner. In order to achieve this, however, it is first necessary to thoroughly understand the complex regulatory networks, involving both pathway-specific and global regulators, which function to control secondary metabolite production in these organisms.

The transformation of distinct "tissues" as part of the developmental program in *Streptomyces* is another area of interest due both to its complexity by prokaryotic standards, and to its connection to secondary metabolite production. Growth of the organism begins with the germination of a single spore, and the subsequent formation of a loose network of branched multigenomic vegetative mycelia (Figure 1.1.1). In response to some unknown signal, probably related to nutritional or environmental stress, growth of vegetative mycelia ceases, and the colony begins to develop aerial hyphae that grow away from the colony surface into the air. This process occurs simultaneously with the hydrolysis of storage compounds such as glycogen in the vegetative mycelia, which may provide the necessary turgor for the erection of the aerial hyphae (Plaskitt & Chater, 1995). As well, the degeneration of vegetative hyphae is observed in specific regions during this phase (Wildermuth, 1970), which is thought to serve as a nutrient source for the developing aerial hyphae (Mendez *et al.*, 1985). This vegetative hyphal cell death has recently been shown to resemble programmed cell death in eukaryotic cells, involving an orderly process of internal cell dismantling that allows removal of the degenerated hyphae without disruption of the colony architecture (Migueluez *et al.*, 1999). Interestingly, the erection of aerial hyphae also coincides with antibiotic production within specific regions of the substrate (vegetative) mycelia, and these compounds are

Figure 1.1.1 *Streptomyces coelicolor* life cycle. Known genes that control specific stages of development are indicated, as well as the stages at which production of the cell-associated undecylprodigiosin antibiotic (indicated in red) and the diffusible actinorhodin antibiotic (indicated in blue) occur. Also indicated are the appearance of coiled and septated aerial hyphae, mature spores, mature wild type colonies and a bald mutant colony. Photos courtesy of the Sanger Institute: *S. coelicolor* Genome Sequencing Project (http://www.sanger.ac.uk/Projects/S_coelicolor/).



proposed to function to protect the degenerating colony from invading microorganisms (Chater & Merrick, 1979). The developmental process then continues with the coiling of the aerial hyphal tips followed by nucleoid partitioning and cross-wall formation at regular intervals to produce unigenomic spore compartments. A second phase of storage compound accumulation is observed in the aerial hyphae as sporulation begins, and the subsequent degradation of glycogen may aid in the rounding of the compartments to form the ovoid spores, while the glycogen degradation products are incorporated into the thickened walls of the mature spores (Chater, 1989). As well, a second phase of hyphal death is observed involving non-sporulating parts of aerial hyphae, suggesting that hyphal death is somehow included in the developmental program of the organism (Migueluez *et al.*, 1999). The final step is the synthesis of a gray spore-associated polyketide pigment, and chains of >50 unigenomic spores (Champness & Chater, 1994) are then ready for dispersal to begin the process again.

The fact that formation of aerial hyphae occurs simultaneously with the production of antibiotics suggests that these two processes share common regulatory elements. Studies addressing the link between morphological and physiological differentiation (antibiotic production) have been done mainly in *Streptomyces coelicolor*, which is the most extensively characterized and the most genetically tractable streptomycete (Bibb, 1996). This organism produces four antibiotics, which allows for the study of both pathway-specific and global regulation of antibiotic production. As well, none of these antibiotics are of clinical significance, which greatly enhances information sharing, and two of these antibiotics (actinorhodin and undecylprodigiosin)

are pigmented, and can thus serve as readily visible indicators of antibiotic production in this organism.

The chromosome of *S. coelicolor* A(3)2 is linear with an origin of replication (*oriC*) located near the centre of the molecule (Bentley *et al.*, 2002), and includes terminal inverted repeats linked to proteins at the free 5' ends (Bao & Cohen, 2001). Replication proceeds bidirectionally from *oriC* (Musialowski *et al.*, 1994), and presumably generates 3' leading strand overhangs at the chromosome ends (Chen, 1996). The 5' recessed ends of the discontinuous strands generated from the removal of the final RNA primers are then extended using the 'end-patching' process, where DNA synthesis is primed by the terminal proteins to fill the gaps (Bao & Cohen, 2001). *S. coelicolor* A3(2) also contains three plasmids, including SCP1, a 350 kb linear plasmid that replicates in a similar manner to the linear chromosome (Chang & Cohen, 1994), and which harbors the genes for the biosynthesis and resistance to the antibiotic methylenomycin (Kinashi *et al.*, 1987; Wright & Hopwood, 1976b). The SCP2 plasmid is a 30 kb circular plasmid that is self-transmissible and capable of mobilizing the chromosome (Bibb & Hopwood, 1981; Bibb *et al.*, 1977), whereas the 17 kb integrative element SLP1 is normally present in the chromosome, but can also be transferred to other streptomycetes where it will undergo autonomous replication (Bibb *et al.*, 1981; Brasch & Cohen, 1993; Omer & Cohen, 1984).

Sequencing of the *S. coelicolor* genome, which is 8.67 Mb in size, has recently been completed, and the G + C content of the genome is 72.12%, with 7825 predicted genes (Bentley *et al.*, 2002). Nearly all of the essential genes (e.g. cell division genes) are located in the core region of the chromosome, whereas non-essential genes (e.g. genes

involved in secondary metabolite production) are found within the chromosome arms. Interestingly, about 12.3% of the predicted protein products are thought to have regulatory function, which supports the notion that the proportion of regulatory genes in an organism increases with the genome size (Bentley *et al.*, 2002).

1.2 Regulation of Spore Formation

The developmental process in which multigenomic aerial hyphae are transformed into chains of unigenomic spores is controlled by a group of genes referred to as the *whi* (white) genes, named for the white appearance of mutant colonies after prolonged incubation due to the absence of the gray spore-associated pigment. Thirteen *whi* genes (*whiA, B, D, E, G-O*) and a gene encoding a sporulation-specific sigma factor (*sigF*) have been identified so far (Chater, 1972; Potuckova *et al.*, 1995; Ryding *et al.*, 1999), all of which are defective at various stages of spore formation. Two of these genes (*whiK* and *whiM*), however, were later reclassified as *bld* genes (see later) due to the aerial mycelium deficient phenotype of null mutations in these genes [(Bibb *et al.*, 2000; Molle & Buttner, 2000); see below]. Nine of these *whi* genes have been placed into a hierarchy based on the sporulation stage that is halted in the mutants: *whiG, whiJ* < *whiA, whiB* < *whiH* < *whiI* < *whiD, sigF, whiE*. The first six genes of this hierarchy (*whiG-whiI*) are further classified as the 'early' *whi* genes and are required for the formation of the sporulation septum, while the final three loci (*whiD, sigF, whiE*) are required for spore maturation, and are therefore referred to as the 'late' *whi* genes. Of these nine sporulation genes, only *whiJ* has not been cloned or characterized.

whiG is required for the earliest stages of sporulation, as mutants of *whiG* display long, straight aerial hyphae that show no signs of coiling or septum formation (Flardh *et al.*, 1999). The product of this gene is an RNA polymerase sigma factor that resembles the motility factors σ^D of *Bacillus subtilis*, and σ^{FliA} of *Salmonella typhimurium* (Chater *et al.*, 1989; Tan & Chater, 1993). Introduction of *whiG* into wild type *S. coelicolor* on a multicopy plasmid resulted in premature and excessive sporulation of aerial hyphae, and also caused ectopic sporulation in vegetative hyphae (Chater *et al.*, 1989), suggesting that the level of σ^{WhiG} is a critical determinant of the decision to undergo sporulation (Flardh *et al.*, 1999). Interestingly, the transcription of *whiG* was shown to occur throughout development, even before aerial hyphae were visible (Kelemen *et al.*, 1996), suggesting that σ^{WhiG} protein levels are controlled by the post-transcriptional regulation of *whiG*, and that a threshold concentration of σ^{WhiG} is necessary to initiate sporulation. An alternate possibility, and one which has been demonstrated for σ^{FliA} of *S. typhimurium* (Ohnishi *et al.*, 1992) is that σ^{WhiG} is post-translationally regulated by an anti-sigma factor protein that binds to σ^{WhiG} and prevents it from associating with core RNA polymerase until the decision to sporulate is reached. Efforts to detect such an anti-sigma factor, however, have proven fruitless.

It has previously been shown using *whi* gene double mutants that *whiG* is epistatic to *whiA*, *whiB* and *whiH*, suggesting that expression of these genes may involve the σ^{WhiG} RNA polymerase (Chater, 1975). Transcriptional analysis of *whiA* and *whiB* indicated that both genes are transcribed from two promoters, one of which is expressed throughout development at low levels, while the other is upregulated during aerial hyphae formation (Ainsa *et al.*, 2000; Soliveri *et al.*, 1992). Expression from either of the *whiA* or *whiB*

promoters, however, was not dependent on *whiG* (Ainsa *et al.*, 2000; Soliveri *et al.*, 1992). On the other hand, transcription of the developmentally regulated *whiA* promoter was reduced in a *whiA* mutant (Ainsa *et al.*, 2000), and increased expression of the developmentally regulated *whiB* promoter was observed in a *whiB* mutant (Chater, 1998), suggesting that WhiA and WhiB may directly or indirectly regulate their own expression. Mutants of *whiA* and *whiB* were found to produce tightly coiled, abnormally long aerial hyphae that had no sporulation septa, and both *whiA* and *whiB* were found to be epistatic to *whiH* (Flardh *et al.*, 1999). It has been proposed that *whiA* and *whiB* define a second critical developmental decision point (after *whiG*) for the commitment to sporulation, and that both genes are required for cessation of aerial hyphae elongation and the initiation of sporulation septation (Flardh *et al.*, 1999).

The *whiA* gene product is a protein of unknown function that has homologs in all other Gram positive bacteria whose genomes have been sequenced to date (Ainsa *et al.*, 2000). WhiB is a small, cytoplasmic protein that is thought to function as a transcriptional regulator (Davis & Chater, 1992) and that belongs to a new family of proteins found only in streptomycetes and other actinomycetes (Soliveri *et al.*, 2000). Interestingly the *whiD* protein product, which functions in the later stages of sporulation along with *sigF* and *whiE*, also belongs to this family of proteins (Molle *et al.*, 2000). *Mycobacterium tuberculosis* was found to contain seven of these proteins that were designated WhmA-G (WhiB-homologs in *mycobacteria*), while at least five genes, designated *wblA-E* (whiB-like), were found in *S. coelicolor* in addition to *whiB* and *whiD* (Soliveri *et al.*, 2000). This family of proteins is characterized by their small size (87-130 amino acids) and by the presence of a C-terminal helix-loop-helix motif that may

function in DNA binding. Also characteristic of these proteins is the presence of four conserved cysteine residues which are arranged in most members of the family as: Cys-X(14-22)-Cys-X(2)-Cys-X(5)-Cys, with the number of X intervening amino acids indicated in brackets (Soliveri *et al.*, 2000). It has been proposed that the cysteine residues may allow the proteins to respond to redox changes through the formation of inter- or intra-molecular disulfide bonds, or through interaction with a redox-active metal atom (Soliveri *et al.*, 2000). In the case of *whiB*, the protein product may function as a sensor of changes in the cytoplasmic redox state in response to aerial hyphae formation itself, or in response to metabolic changes that occur as aerial hyphae formation slows down (Chater, 1998; Chater, 2001). *WhiD*, on the other hand, may sense redox changes that occur in the later stages of sporulation.

A connection between early sporulation genes was made by the finding that expression of both *whiH* and *whiI* is dependent on σ^{WhiG} (Ainsa *et al.*, 1999; Ryding *et al.*, 1998). Moreover, transcriptional analysis indicated that both *WhiH* and *WhiI* negatively autoregulate expression from their own promoters, and *WhiI* significantly represses transcription of the *whiH* promoter, while *WhiH* affected *whiI* gene expression to a moderate extent (Ainsa *et al.*, 1999; Ryding *et al.*, 1998). *whiH* was found to encode a protein showing similarity to members of the GntR family of regulatory proteins, of which several of the members have been shown to bind DNA and function as repressors of genes involved in carbon metabolism (Ryding *et al.*, 1998). Subsequent alleviation of this repression occurs in most cases through interaction of the regulatory protein with an acidic carbon metabolite. The N-terminal region of *WhiH* contains a putative helix-turn-helix motif characteristic of other regulatory proteins of the family, and mutational

analysis indicated that this motif is important for WhiH function (Ryding *et al.*, 1998). Also, transcriptional analysis of *whiH* and *whiI* demonstrated that both genes are developmentally regulated, with expression starting when aerial hyphae become visible, and maximal levels occurring during sporulation (Ainsa *et al.*, 1999; Ryding *et al.*, 1998). It is therefore possible that WhiH may also be regulated by an unknown carbon metabolite whose concentration changes during development, and that interaction with this metabolite would result in derepression of *whiH* and *whiI*, as well as genes that are directly required for sporulation. In this manner, WhiH would act as a sensor of the metabolic or physiological state of the aerial hyphae, and transmission of this information would lead to expression of genes required for septum formation. Alternatively, WhiH could function to negatively regulate another repressor protein that would normally prevent sporulation, or it could even act as both a repressor and an activator of gene expression.

The *whiI* protein product is an atypical member of the FixJ-subfamily of response regulators whose members normally function along with sensor kinase proteins in two-component signal transduction systems in prokaryotes (Ainsa *et al.*, 1999; Hackenbeck & Stock, 1996). Typically, the sensor kinase responds to some signal by phosphorylating the response regulator on a conserved aspartate residue, which in turn causes the regulator to induce or repress the expression of genes of a particular regulon. While the gene encoding the sensor kinase is usually located next to the cognate response regulator, examination of the *S. coelicolor* genome sequence revealed that a sensor kinase gene was not present within the vicinity of *whiI*. Also, WhiI lacks two of the highly conserved residues in the conventional phosphorylation pocket, suggesting that it may not be

phosphorylated like the other regulator proteins of the same family (Ainsa *et al.*, 1999). As suggested for WhiH, WhiI may alternatively function as a sensor of an unknown intracellular signal, either by interacting directly with a ligand, or by interacting with another regulatory protein. Assuming that WhiH and WhiI do in-fact function as sensors, and given the fact that WhiH and WhiI regulate transcription of each other's promoters, it is likely that more than one signal is required in order for expression of WhiH and WhiI-dependent genes to occur.

It has previously been shown that the cell division protein FtsZ is dispensible for colony growth, but is required for the sporulation of aerial hyphae in *S. coelicolor* (McCormick *et al.*, 1994). Immunofluorescence microscopy revealed that FtsZ assembles into long, regularly spaced, ladder-like structures in sporulating aerial hyphae, which represents the earliest known molecular indicator of sporulation in *Streptomyces* (Schwedock *et al.*, 1997). Similarly with cell division in unicellular bacteria, each FtsZ ring is thought to represent the site at which a sporulation septum will form. Interestingly, the formation of this ladder requires expression of *ftsZ* from one of its three promoters (*ftsZP2*), and upregulation of this promoter in developing aerial hyphae requires the presence of the early sporulation genes *whiA*, *B*, *H*, *I* and *J* (Flardh *et al.*, 2000; Schwedock *et al.*, 1997). Regulation of *ftsZP2* was also found to be dependent on *whiG*, however this dependence may be manifested through the WhiG targets WhiH and/or WhiI, as analysis of the *ftsZP2* sequence revealed that it does not match the σ^{WhiG} consensus (Flardh *et al.*, 2000). Given the similarity of the *whiH* mutant phenotype with the phenotype of a strain lacking *ftsZP2* [condensation of nucleoids into discrete areas,

reduced formation of gray spore pigment, few if any sporulation septa; (Flardh *et al.*, 2000)], it seems possible that WhiH may activate *ftsZP2*.

Mutants of the late sporulation gene *sigF* produce small, unpigmented, deformed spores that have thin spore walls, uncondensed DNA, and are sensitive to detergents, indicating that *sigF* is required for the expression of genes involved in the late stages of spore maturation (Potuckova *et al.*, 1995). The protein product of *sigF* is a second sporulation-specific RNA polymerase sigma factor that shows the greatest similarity to σ^B of *Bacillus subtilis*, which is involved in stationary phase gene expression and in the general stress response (Benson & Haldenwang, 1993c; Boylan *et al.*, 1993a; Boylan *et al.*, 1993b; Potuckova *et al.*, 1995). Transcriptional analysis indicated that the *sigF* promoter is both developmentally and spatially regulated, as expression of *sigF* was limited to developing and mature spore chains, and was not detected in vegetative hyphae or during aerial hyphae formation (Kelemen *et al.*, 1996; Sun *et al.*, 1999). Transcription was also found to be dependent on all of the early *whi* genes (*A,B,G,H,I,J*), however the dependence on *whiG* appeared to be indirect, as purified RNAP- σ^{WhiG} could not recognize the *sigF* promoter in *in vitro* run-off transcription assays, and the *sigF* promoter sequence did not conform to the RNAP- σ^{WhiG} consensus sequence (Kelemen *et al.*, 1996). It is possible that a third unidentified sporulation-specific sigma factor which is itself dependent on WhiG is responsible for *sigF* transcription, or that the effect of WhiG is mediated through the putative transcriptional regulators WhiH and/or WhiI. In *Streptomyces aureofaciens*, which has several of the same developmental regulatory proteins found in *S. coelicolor*, it has been shown that the *sigF* promoter is bound by two unknown proteins, one (designated RsfA) that binds during the early stages of

differentiation and possibly functions as a repressor, and another (designated RsfB) that binds during sporulation separation, and may serve as an activator (Homerova *et al.*, 2000). The identification of these proteins will undoubtedly provide useful information as to the regulation of *sigF* expression in *S. coelicolor*.

Transcriptional analysis of the *whiE* locus has indicated that as with *ftsZP2* and *sigF*, expression is dependent on all of the early *whi* genes. *whiE* consists of a cluster of eight genes, seven of which (ORFI-VII) are transcribed from the *whiEP1*, while a single gene (ORFVIII) is divergently transcribed from the *whiEP2* promoter (Davis & Chater, 1990; Kelemen *et al.*, 1998). The genes at this locus specify proteins for the biosynthesis of the gray spore-associated pigment, as mutants of *whiE* are able to produce morphologically normal spores that appear white due to the absence of the spore pigment (Chater, 1972; McVittie, 1974). Expression from both *whiE* promoters is developmentally regulated, and one promoter (*whiEP2*) is dependent on *sigF*, which suggests that expression from this promoter is also spatially regulated (Kelemen *et al.*, 1998).

Recently, a new family of five paralogous genes has been identified in *S. coelicolor* that appear to be specific for *Streptomyces* spp., and that are thought to be required for morphological differentiation in these organisms (Kormanec & Sevcikova, 2002). The best characterized gene of this family, *ssgA*, has been shown in both *S. coelicolor* and *S. griseus* to be required for sporulation (Jiang & Kendrick, 2000; van Wezel *et al.*, 2000), and overexpression of *ssgA* in *S. coelicolor* resulted in an increase in both the frequency and thickness of sporulation septa, as well as an increase in the thickness of the hyphae themselves (van Wezel *et al.*, 2000). Interestingly, it was found

that the sporulation deficiency of *S. coelicolor* and *S. griseus ssgA* mutant strains was conditional, as low levels of spore production could be observed on certain media after prolonged incubation. This is in contrast to previously characterized *whi* mutant strains (excluding *whiE* mutants), which do not produce mature spores on any type of media. Moreover, disruption of *ssgA* was also found to significantly reduce the production of the blue pigmented antibiotic actinorhodin by *S. coelicolor*, whereas mutations in the *whi* genes do not typically affect secondary metabolite production. Thus *ssgA* (and possibly the other four paralogs) represents a novel *whi* gene in *S. coelicolor* that may provide a link between secondary metabolism and sporulation, and it is speculated that it may encode a protein that directly interacts with one or more proteins of the cell division assembly (van Wezel *et al.*, 2000).

1.3 Regulation of Aerial Mycelium Formation

While the *whi* genes, *sigF*, *ftsZ* and *ssgA* are involved in the transformation of multigenomic aerial hyphae into chains of mature spores, the formation of aerial hyphae itself is controlled by a separate group of genes known as the *bld* (bald) genes. These genes are named for the smooth, shiny, 'bald' appearance of mutant colonies on rich medium in comparison to the fuzzy appearance of the wild type strain (see Figure 1.1.1). Thirteen *bld* genes (*bldA-D*, *F-N*) have been identified so far (Champness, 1988; Harasym *et al.*, 1990; Merrick, 1976; Nodwell *et al.*, 1996; Nodwell *et al.*, 1999; Passantino *et al.*, 1991; Willey *et al.*, 1993), and mutations in many of these genes result in defects in production of at least one of the four antibiotics produced by *S. coelicolor*,

thus providing genetic evidence for the link between morphological and physiological differentiation in these organisms. For example, *bldA*, *B*, *D*, and *I* are all defective in the production of all four antibiotics, which include actinorhodin, a blue diffusible pigment (under basic conditions), undecylprodigiosin, a red cell-associated pigment, methylenomycin, a plasmid-encoded antibiotic, and calcium-dependent antibiotic (CDA) (Harasym *et al.*, 1990; Hopwood & Wright, 1983; Merrick, 1976). *bldG* and *bldH* are known to be defective in production of three of the antibiotics (actinorhodin, undecylprodigiosin, methylenomycin) (Champness, 1988), while a *bldF* mutant was only able to produce undecylprodigiosin (Passantino *et al.*, 1991), and *bldC* mutants are only defective in the production of CDA (Hopwood & Wright, 1983). *bldK* mutants were found to be defective in actinorhodin production when grown on rich medium, however this defect was conditional, as antibiotic production was unimpaired when the strain was grown on glucose minimal medium (Nodwell *et al.*, 1996).

The metabolic status of a growing colony is thought to play an important role in the decision to differentiate, as it has been observed that mutations in many of the *bld* genes result in defective carbon catabolite repression of metabolic operons (Pope *et al.*, 1996). Specifically, Pope *et al.* (1996) showed that the promoter of the galactose operon is constitutively expressed when certain mutants are grown under conditions where it would normally be repressed. This observation, along with the finding that aerial hyphae formation can be partially or even completely restored in many *bld* mutants when they are grown on a poor carbon source such as mannitol (Chater, 1989; Nodwell *et al.*, 1996), has led to the proposal that the inability of *bld* mutants to differentiate may be a secondary consequence of their inability to sense and/or signal starvation. In conjunction

with this idea, the intracellular starvation signal ppGpp has been suggested to be important for induction of differentiation under nitrogen limiting conditions, as a constructed mutant of the ppGpp synthase gene *relA* failed to produce antibiotics and displayed a marked delay in the onset and extent of morphological differentiation (Chakraborty & Bibb, 1997).

More recently, a connection between metabolism and development has been implicated through the construction of a mutant for the adenylate cyclase gene, *cya*, which catalyzes the production of cAMP, a molecule that is involved in the activation of genes involved in the utilization of alternative carbon sources in *E. coli* (Susstrunk *et al.*, 1998). While the role of cAMP in glucose catabolite repression in *Streptomyces* is unclear, the *cya*⁻ mutant was unable to produce aerial hyphae or pigmented antibiotics on glucose minimal medium, and was found to irreversibly acidify the surrounding medium due to the accumulation of organic acids (Susstrunk *et al.*, 1998). Similar observations were also made for mutants of the citrate synthase gene, *citA*, which acts at the junction of the glycolytic and tricarboxylic acid (TCA) pathways, and of the aconitase gene, *acoA*, which catalyzes the isomerization of citrate to isocitrate in the TCA cycle (Viollier *et al.*, 2001a; Viollier *et al.*, 2001b). Acidification of the growth medium by organic acids generated from glucose has been observed for wild type *S. coelicolor*, however this was subsequently followed by neutralization of the medium, either by the uptake and metabolism of the organic acids, and/or by the excretion of basic compounds (Susstrunk *et al.*, 1998). In the case of the *cya*⁻, *citA*⁻ and the *acoA*⁻ mutants, the developmental defects were suppressed by growing the mutant strains on buffered media, indicating that the ability to neutralize the surrounding medium is necessary for proper development.

Interestingly, several *bld* mutants were also shown to irreversibly acidify the growth medium, however this cannot solely explain the developmental defects of these mutants, as growth on buffered media did not restore the wild type phenotype to these strains (Susstrunk *et al.*, 1998).

On rich medium, the erection of aerial hyphae by *Streptomyces* spp. is associated with the production and secretion of a small, hydrophobic, non-ribosomally-synthesized protein called SapB [Sap, spore-associated protein; (Willey *et al.*, 1993; Willey *et al.*, 1991)]. This protein functions in a similar manner to fungal hydrophobins in that it reduces the surface tension of the aqueous environment at the colony surface, and thereby allows the aerial hyphae to break free and grow into the air (Tillotson *et al.*, 1998; Wosten & Willey, 2000). Interestingly, it has been shown that many of the *bld* mutants are defective in the production of SapB, and restoration of aerial structures by these mutants occurs when they are grown on rich media next to a SapB-producing strain, or when purified SapB protein is added to the colonies (Willey *et al.*, 1993; Willey *et al.*, 1991). The exogenous addition of SapB, however, does not lead to restoration of true aerial hyphae, since they remain undifferentiated, indicating that there are other processes involved in morphogenesis that are regulated by the *bld* genes in addition to SapB production (Tillotson *et al.*, 1998; Willey *et al.*, 1993). Moreover, the production of aerial hyphae and spores can also occur in the absence of SapB when wild type *S. coelicolor* and certain *bld* mutants are grown on minimal medium containing mannitol as a carbon source (Champness, 1988; Chater, 1989; Willey *et al.*, 1991), suggesting that there is at least one additional pathway for aerial hyphae formation that does not involve SapB or certain *bld* genes.

Recently a novel family of secreted hydrophobic proteins has been identified in *S. coelicolor* whose members are also thought to allow aerial hyphae to break the surface tension of the colony surface and grow into the air (Elliot *et al.*, 2003b). This chaplin protein family (*S. coelicolor* hydrophobic aerial proteins) consists of eight members, all of which contain a highly conserved hydrophobic 'chaplin' domain, and it is proposed that these proteins polymerize through interaction between the domains to form a hydrophobic layer which is anchored to the cell wall. A separate set of proteins known as the rodlin proteins (RdlA, RdlB) have also been shown to coat the surface of aerial hyphae and spores, forming a basketwork of paired rodlet structures (Claessen *et al.*, 2002). In this case, the proteins are thought to be involved in the attachment of aerial hyphae to hydrophobic surfaces, and not in the erection of aerial hyphae, since disruption of the rodlin genes did not affect the growth or the overall hydrophobicity of aerial mycelia.

An interesting feature of many *bld* mutants is their ability to either complement a second *bld* mutant or to be complemented by a second mutant, when the two are grown in close proximity on rich medium (Willey *et al.*, 1993). The complementation that takes place, which results in restoration of SapB production, aerial hyphae formation and sporulation, is invariably unidirectional, with one mutant acting as the 'donor' strain and the other acting as the 'recipient' strain. Based on experiments involving a number of *bld* mutants, the following hierarchy of *bld* genes has been established: *bldJ* < *bldK*, *bldL* < *bldA*, *bldH* < *bldG* < *bldC* < *bldD*, *bldM*, where each mutant can complement all of the *bld* mutants to the left, but not to the right of it. Thus *bldD* and *bldM* mutants can complement all of the other *bld* mutants, while *bldJ* is complemented by all other *bld*

mutants. In the case of *bldH* and *bldA* mutants, they can complement *bldK* and *J* mutants and are themselves complemented by *bldG, C, D* and *M* mutants, but they are unable to complement each other, and therefore they have been placed into the same complementation group. An interpretation of this extracellular complementation hierarchy is that SapB production and aerial mycelium formation are regulated by a signaling cascade that involves at least five extracellular signals, and that the *bld* genes themselves encode these signals, or are directly or indirectly responsible for their synthesis or for their uptake into the organism (Willey *et al.*, 1993). Evidence for such an idea comes from studies of the *bldK* locus, which consists of five genes encoding components of an ATP-binding cassette oligopeptide permease (Nodwell *et al.*, 1996), and of a *bldJ* (formerly *bld261*)-dependent signaling peptide that is proposed to be the first signal in the cascade and is thought to be imported by the BldK oligopeptide permease (Nodwell & Losick, 1998). On the other hand, the existence of such a simplified linear cascade cannot be the only explanation for the role of the *bld* genes in the regulation of development, as a number of *bld* genes (*bldB*, *bldI*, *bldN*) do not fit neatly into this hierarchy. Furthermore, this model does not address the defect in antibiotic production observed with several of the *bld* mutants, a defect that is not rectified by growing pairs of *bld* mutants in close proximity (Willey *et al.*, 1993). Despite these complications, however, a number of new mutants defective in morphological differentiation have been identified recently, many of which fall into the *bldC*, *bldD/M* and *bldK* complementation groups (Nodwell *et al.*, 1999), thus further emphasizing the complexity involved in the regulation of aerial hyphae formation.

In an effort to better understand all of the observations and to piece together the factors that either trigger or regulate differentiation, a number of *bld* genes have been cloned, sequenced and characterized. Such investigations have revealed a variety of gene products, of which most appear to operate at the level of transcriptional, translational or post-translational regulation. The best characterized gene to date, *bldA*, encodes the only leucyl tRNA that can efficiently translate the UUA codon which is quite rare in the high G+C content DNA of *Streptomyces* (Lawlor *et al.*, 1987; Leskiw *et al.*, 1991b).

Mutations in *bldA* have been shown to abolish aerial hyphae formation and antibiotic production, however vegetative growth appeared to be unaffected, suggesting that genes necessary for growth and primary metabolism are devoid of TTA codons (Merrick, 1976). This was supported by the examination of a number of gene sequences, which indicated that TTA codons are confined to genes involved in aerial hyphae formation, antibiotic biosynthesis and resistance, and pathway-specific regulation of antibiotic production (Leskiw *et al.*, 1991a; Trepanier, 1999). For example, TTA codons have been found in the *actII*-ORF4 and the *redZ* genes, which function in the pathway-specific activation of the pigmented antibiotics actinorhodin and undecylprodigiosin, respectively [(Fernández-Moreno *et al.*, 1991; Guthrie *et al.*, 1998; White & Bibb, 1997); see below]. Transcriptional analysis indicated that the *bldA* promoter is active at all stages of growth, however accumulation of the mature, processed tRNA occurs later in development, supporting the notion that the *bldA*-specific tRNA is involved in the translational regulation of genes involved in morphological and physiological differentiation (Leskiw *et al.*, 1993). Interestingly, an anti-sense RNA transcript originating from within the *bldA* gene was detected during vegetative growth, and it is intriguing to speculate that such an

anti-sense transcript may be involved in the temporal processing of the *bldA*-specific transcript (Leskiw *et al.*, 1993).

While it has been shown that *bldA* is required for the efficient translation of TTA codons in *actII*-ORF4 and *redZ* (Gramajo *et al.*, 1993; White & Bibb, 1997), as well as in the reporter genes *ampC*, *lacZ* and *carB* (Leskiw *et al.*, 1991b), there is also evidence that under certain conditions, TTA codons can be translated in the absence of *bldA*. For example, the TTA-containing *aad* and *hyg* reporter genes, conferring resistance to spectinomycin and hygromycin, respectively, were found to be only partially dependent on *bldA* for expression (Leskiw *et al.*, 1991b). Furthermore, translation of *ccaR*, encoding the pathway-specific regulator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus*, was the same in a *bldA* mutant of *S. clavuligerus* and *S. coelicolor* as it was in the corresponding wild type strains, suggesting that the TTA codon in *ccaR* was being efficiently mistranslated in the *bldA* mutants by a noncognate tRNA (Trepanier *et al.*, 2002). One possible explanation for this is that the frequency of mistranslation of TTA codons depends on the context of the TTA codon, particularly the nucleotide found 3' to the codon. This conclusion comes from the examination of a number of TTA-containing genes, where TTA codons followed by a G or A were generally found in genes that appeared to be mistranslated, whereas codons followed by a C or T were found in *bldA*-dependent genes (Trepanier *et al.*, 2002). Thus the presence of a TTA codon in a gene may not necessarily mean that it is absolutely dependent on *bldA* for expression.

Of the *bld* mutants that have been studied, *bldB* mutants have the most pleiotropic phenotypes. In addition to being completely defective in the production of aerial hyphae

and antibiotics, *bldB* mutants are one of the few *bld* mutants that cannot sporulate when grown on poor carbon sources such as mannitol (Merrick, 1976; Pope *et al.*, 1996). As well, they are defective in carbon catabolite repression of the galactose, glycerol and agarose utilizing operons as opposed to being defective in repression of just the galactose operon (Pope *et al.*, 1996), and *bldB* does not fit into the extracellular complementation cascade described above. Cloning of *bldB* revealed that it encodes a small protein that shows similarity to AbaA and WhiJ of *S. coelicolor*. Little is known about AbaA and WhiJ except that they are involved in antibiotic production and sporulation, respectively (Eccleston *et al.*, 2002; Harasym *et al.*, 1990). Transcriptional analysis of *bldB* revealed that it is expressed at low levels during vegetative growth, and is upregulated at the initiation of development (Pope *et al.*, 1998). However, this temporal pattern was abolished in a *bldB* mutant background, and expression levels were much higher, suggesting that BldB negatively autoregulates expression from its own promoter. This is supported by the observation that BldB contains a possible helix-turn-helix motif in the C-terminal region, and that it interacts with itself to form a potential dimer, suggesting that it may function as a DNA binding protein (Eccleston *et al.*, 2002; Pope *et al.*, 1998). Interestingly, it has been observed that the phenotype of the *bldB* mutant is the same as that of a glucose kinase (*glkA*) mutant in terms of relief of glucose repression (Pope *et al.*, 1998). GlkA is required for glucose-mediated catabolite repression of some genes in *S. coelicolor* independent of its kinase activity (Kwakman & Postma, 1994), and since a DNA binding motif is not associated with GlkA, it is possible that a BldB-GlkA complex may interact with DNA regulatory regions to control expression of catabolite-controlled promoters, or of genes required for catabolite repression. In this way, BldB may serve as

a sensor of signals that are required to evaluate the nutritional status of the colony, and the observed pleiotropic phenotype of *bldB* mutants may be the result of the inability of these mutants to sense starvation conditions. Alternatively, BldB may have other functions in the cell that are more directly involved with morphogenesis in addition to carbon catabolite repression.

Analysis of the *bldD* protein product revealed that it did not show similarity to any known proteins in the database, however a putative helix-turn-helix motif in the C-terminal region and an N-terminal HTH-3 motif suggested that it may function as a DNA binding protein (Elliot *et al.*, 1998; Elliot *et al.*, 2001). In support of this, expression of *bldD* was found to be much higher and was detected throughout growth in a *bldD* mutant background compared to the wild type strain, where expression levels decreased once aerial hyphae were visible (Elliot *et al.*, 1998). As well, BldD was shown to be able to bind to its own promoter as a dimer (Elliot & Leskiw, 1999; Elliot *et al.*, 2003a), indicating that BldD functions as a negative regulator of its own expression. Interestingly, BldD has also been shown to directly repress the expression of a putative transcription factor (*bdtA*) as well as other genes involved in differentiation such as *whiG* and *bldN*, which encode sigma factors required for initiation of sporulation and aerial hyphae formation, respectively [(Elliot *et al.*, 2001); see below]. The dependence of *whiG* on *bldD* represents the first link identified between *bld* and *whi* genes, however this dependence needs to be further examined, since as previously mentioned, *whiG* has been shown to be transcribed at constant levels throughout growth. In the case of *bldN*, expression in the wild type strain is repressed until aerial hyphae begin to appear (Bibb *et al.*, 2000; Elliot *et al.*, 2001), which suggests that BldD functions to prevent the

premature expression of genes that are required for development. This function is similar to that described for AbrB and SinR of *B. subtilis*, which are involved in controlling the expression of sporulation-specific sigma factors (Strauch & Hoch, 1993). An additional target of BldD is the developmentally regulated *sigHP2* promoter, which transcribes the SigH sigma factor associated with the stress response in *S. coelicolor* (Kelemen *et al.*, 2001). As with *bldN*, expression from the *sigHP2* promoter is repressed during vegetative growth, and the control of this promoter by BldD suggests that the programs responsible for development and for the stress response may be interconnected in *S. coelicolor* (Kelemen *et al.*, 2001).

The mechanism by which relief of BldD repression occurs is currently unknown, however it has been speculated based on studies of SinR that BldD activity may be inhibited by interaction with either another protein or with a small molecule cofactor (Elliot *et al.*, 2001). It is also unknown why the single *bldD* point mutant and a *bldD* deletion mutant are defective in aerial hyphae formation and antibiotic production when BldD appears to repress genes involved in development (Elliot *et al.*, 1998; Elliot *et al.*, 2003a; Merrick, 1976). It is possible that the observed mutant phenotype is simply due to the premature and ectopic expression of developmental genes, or that BldD also functions as a transcriptional activator of developmental genes. Support for the latter idea comes from recent DNA microarray data suggesting that a putative sigma factor (2SCK8.34), which may or may not be involved in development, is induced in response to overexpression of BldD. In addition, binding of purified BldD to the 2SCK8.34 promoter has been demonstrated *in vitro* (C. Galibois, unpublished).

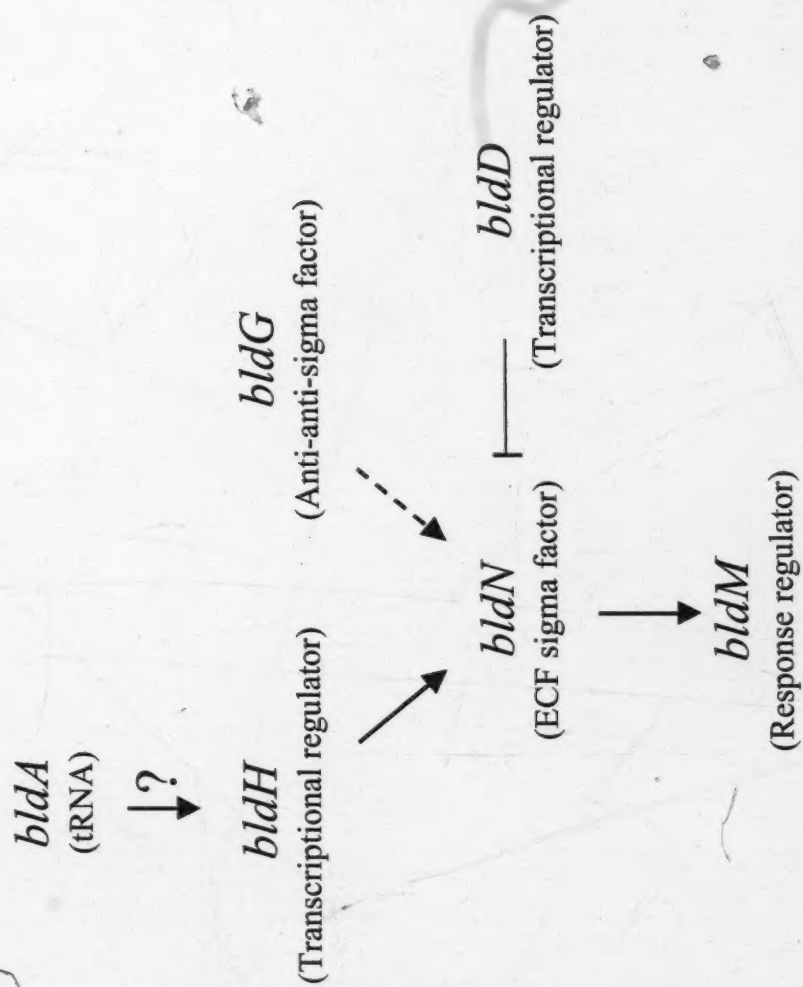
The *whiK* and *whiN* loci were originally isolated in a screen of (NTG)-induced *whi* mutants, since point mutations in both genes resulted in defective sporulation (Ryding *et al.*, 1999). Constructed null mutants of *whiK* and *whiN*, however, were unable to produce aerial hyphae, prompting researchers to rename *whiK* as *bldM*, and *whiN* as *bldN* (Bibb *et al.*, 2000; Molle & Buttner, 2000). *bldM* encodes a typical member of the FixJ-subfamily of two-component response regulators, with a putative C-terminal helix-turn-helix DNA binding domain, and a putative N-terminal phosphorylation pocket (Molle & Buttner, 2000). As previously mentioned, response regulator proteins are typically controlled by phosphorylation of a conserved aspartate residue by a cognate sensor kinase. However, it is unclear as to whether BldM is also regulated by phosphorylation, as mutation of the putative phosphorylation site did not affect BldM function, and purified BldM could not be phosphorylated *in vitro* by small-molecule phosphodonors (Molle & Buttner, 2000). The *bldN* gene encodes an extracytoplasmic function (ECF)-RNA polymerase sigma factor that has an unusual N-terminal extension of 86 amino acids that is absent in other sigma factors (Bibb *et al.*, 2000). Recent studies have shown that BldN is synthesized as a pro-protein with the N-terminal extension, and that this extension is subsequently removed by proteolysis by an unknown protein (Bibb & Buttner, 2003). Since the appearance of the mature BldN protein coincides with aerial hyphae formation, it has been suggested that the BldN pro-protein is an inactive form of the protein, and that post-translational processing of BldN serves as a means of regulating the activity of BldN during development (Bibb & Buttner, 2003).

The *bldM* and *bldN* protein products are similar to the *bldC* and *bldL* protein products in that they do not appear to be required for the production of pigmented

antibiotics in *S. coelicolor* (Bibb *et al.*, 2000; Chater, 1989; Molle & Buttner, 2000; Nodwell *et al.*, 1999). Also, *bldM* and *bldN* null mutants, like *bldB* mutants, cannot synthesize aerial hyphae and spores when grown on a poor carbon source like mannitol, and like *bldB*, *bldN* does not fit into the extracellular complementation cascade proposed by Willey *et al.* (1993). A number of links between different *bld* genes have recently been made through transcriptional analyses of *bldM* and *bldN* (Figure 1.3.1). As previously mentioned, transcription of *bldN* is developmentally regulated, and this regulation appears to be partially dependent on the BldD repressor protein. Interestingly, the expression of *bldN* is also dependent on *bldH*, which encodes the *S. coelicolor* orthologue of the AdpA transcriptional regulatory protein of *S. griseus* [(Chater & Horinouchi, 2003); see below]. *bldH* in turn contains a TTA codon, which may be dependent on the *bldA*-specific tRNA. However, *bldA* is not required for *bldN* expression when strains are grown on rich medium (Bibb *et al.*, 2000), which suggests that the *bldA*-dependent regulation of *bldH* must not exist under those growth conditions. In addition, the transcription of *bldN* is dependent on *bldG* (Bibb *et al.*, 2000), the gene that is the subject of this thesis, and which encodes a protein showing similarity to the SpoIIAA and RsbV anti-anti-sigma factors of *B. subtilis* (see later). As discussed below, the *Bacillus* SpoIIAA and RsbV proteins function along with anti-sigma factor proteins to regulate sigma factors involved in sporulation and the general stress response. Since BldN does not direct transcription from its own promoter, the influence of *bldG* on *bldN* transcription must involve another sigma factor required for aerial hyphae formation. BldN in turn directs transcription from the *bldMP1* promoter, which as a result is also dependent on *bldH*, *bldG* and *bldD* (Bibb *et al.*, 2000). It therefore appears

Figure 1.3.1 Interactions between various *bld* genes in *S. coelicolor*. Arrows represent positive regulatory dependence, with solid arrows indicating a known or predicted direct dependence, and broken arrows representing a known or predicted indirect dependence.

The line between *bldD* and *bldN* represents a direct, negative, regulatory dependence. (?) signifies a dependence that is predicted, but has not yet been established.



that *bldN* may represent a branch point in a cascade at which other *bld* genes (*bldH*, *G*, *D*) converge (Figure 1.3.1). In addition, these findings also indicate the regulatory network controlling aerial hyphae formation is much more complex than a simple linear *bld* gene cascade.

The *ram* (rapid aerial mycelium) gene cluster, like *bldM*, *bldN*, *bldC*, and *bldL*, has been implicated in aerial hyphae formation, but has no apparent effect on secondary metabolite production. This gene cluster is homologous to the *amf* cluster of *Streptomyces griseus*, which is also involved in aerial hyphae formation in that organism (Ueda *et al.*, 1998; Ueda *et al.*, 1993). Three of the *ram* genes, *ramA*, *ramB* and *ramR*, were originally discovered in a screen for *S. coelicolor* clones that accelerated aerial mycelium formation in *Streptomyces lividans* (Ma & Kendall, 1994), and subsequently two additional genes, *ramC* and *ramS*, which are located upstream of *ramA*, have been identified (Keijser *et al.*, 2000). Mutational studies suggested that only *ramC*, *ramS* and *ramR* are essential for normal development of aerial hyphae (Nguyen *et al.*, 2002; O'Connor *et al.*, 2002), however all of the *ram* genes appear to play a role in the growth of aerial hyphae, since the progressive inclusion of adjacent *ram* genes on plasmids increasingly accelerated the formation of aerial hyphae in a *bld* mutant strain (Nguyen *et al.*, 2002). Analysis of the *ram* gene products suggested that *ramC* encodes a putative Ser/Thr kinase that is localized in the membrane, while *ramS* encodes a small, putative signaling peptide, and *ramA* and *ramB* encode ATP-binding membrane transport proteins which may serve as the primary exporters of the RamS peptide (Hudson *et al.*, 2002; Keijser *et al.*, 2000; Nguyen *et al.*, 2002; O'Connor *et al.*, 2002). Expression from the *ramC* promoter [which may in turn control expression of *ramS*, *A* and *B*; (Keijser *et al.*,

2000; Nguyen *et al.*, 2002)] was shown to be limited to vegetative hyphae and pre-septational aerial hyphae, and RamC protein was detected in substrate hyphae and was absent from spores, suggesting that RamC is required for aerial hyphae formation, but not for sporulation (O'Connor *et al.*, 2002). Interestingly, the production of RamC protein was abolished in a *bldD* and a *cprA* mutant, where *cprA*, as discussed below, encodes a putative transcriptional regulator that may function as a positive activator of both secondary metabolite production and sporulation (Onaka *et al.*, 1998). This dependence on *bldD* and *cprA*, however, is most likely indirect in both cases, since a BldD binding site was not found within the *ramC* promoter region, and binding of purified CprA to the *ramC* promoter *in vitro* could not be demonstrated (O'Connor *et al.*, 2002).

Alternatively, the inability of CprA to interact with the *ramC* promoter may be due to the absence of a component in the *in vitro* system that is required for protein binding.

ramR encodes a putative response regulator that resembles members of the FixJ subfamily of response regulatory proteins (Ma & Kendall, 1994; Nguyen *et al.*, 2002), and mutational analysis suggested that RamR activity is regulated by phosphorylation, although the kinase responsible for phosphorylation is unknown (Nguyen *et al.*, 2002). Overexpression of RamR was able to restore aerial hyphae formation and SapB production to all classical *bld* mutants tested, and production of RamC was severely defective in an *ramR* mutant, suggesting that RamR regulates the expression of *ramC* (and possibly the other *ram* genes) (Nguyen *et al.*, 2002; O'Connor *et al.*, 2002). This was confirmed by demonstrating that purified RamR could bind specifically to the *ramC* promoter *in vitro* (Nguyen *et al.*, 2002; O'Connor *et al.*, 2002). Thus RamR appears to be a key activator of development, and it has been suggested that it functions similarly to

SpoOA of *Bacillus subtilis* in that it senses and integrates a number of physiological signals to ensure that conditions are right before initiating development in *S. coelicolor* (Nguyen *et al.*, 2002).

1.4 Regulation of Antibiotic Production

As previously mentioned, *S. coelicolor* produces four different antibiotics, including actinorhodin (Act) (Wright & Hopwood, 1976a), undecylprodigiosin (Red) (Rudd & Hopwood, 1980), methylenomycin A (Mmy) (Wright & Hopwood, 1976b) and calcium-dependent antibiotic (CDA) (Hopwood & Wright, 1983). Actinorhodin is a blue, diffusible polyketide molecule that is produced by an enzyme that is evolutionarily related to bacterial fatty acid synthases (Hopwood & Sherman, 1990). The genes required for Act biosynthesis, resistance and export are found clustered together on the chromosome (Fernández-Moreno *et al.*, 1991; Malpartida & Hopwood, 1984), an arrangement that is generally observed for antibiotic genes in *Streptomyces* spp. (Bibb, 1996). Included in the *act* gene cluster is *actII*-ORF4, encoding the pathway-specific regulator of Act production in *S. coelicolor* (Fernández-Moreno *et al.*, 1991). This protein belongs to a family of regulatory proteins known as the SARPs (*Streptomyces Antibiotic Regulatory Proteins*), which are characterized by the presence of an N-terminal OmpR-like DNA binding domain that is thought to bind promoter regions at heptameric repeats (Wietzorrek & Bibb, 1997). Expression studies have shown that transcription of *act* genes does not occur in an *actII*-ORF4 mutant (Hallam *et al.*, 1988), and that extra copies of *actII*-ORF4 cloned into wild type *S. coelicolor* causes Act overproduction (Chater, 1990; Gramajo *et al.*, 1993), suggesting that ActII-ORF4 functions as a

transcriptional activator of the *act* biosynthetic genes. This has been further extended by the demonstration that purified ActII-ORF4 can bind to specific regions within the *act* promoters (Arias *et al.*, 1999), and that transcription of some *act* biosynthetic genes, and the subsequent production of Act, closely follows the increase in expression of *actII*-ORF4 that occurs during transition from exponential to stationary phase in liquid culture (Gramajo *et al.*, 1993). In addition, the introduction of extra copies of *actII*-ORF4 results in Act production during exponential phase, suggesting that the growth-phase dependence of Act production is largely dependent on the temporal regulation of *actII*-ORF4 (Gramajo *et al.*, 1993). Transcriptional regulation of *actII*-ORF4 is known to involve the starvation signaling molecule ppGpp (Hesketh *et al.*, 2001), and *actII*-ORF4 also contains a *bldA*-dependent TTA codon, which further subjects this gene to regulation at the level of translational control (Fernández-Moreno *et al.*, 1991).

The second pigmented antibiotic produced by *S. coelicolor*, referred to as undecylprodigiosin (Red), is in reality a mixture of three different prodigionines, including undecylprodigiosin, butylcycloheptylprodigiosin, and dipyrrolyldipyrromethane, of which undecylprodigiosin is the major constituent (Tsao *et al.*, 1985). As with actinorhodin production, the synthesis of the cell-associated Red pigment occurs in a growth phase-dependent manner, with production occurring in liquid cultures in the transition and stationary phases (Takano *et al.*, 1992), and coinciding with the formation of aerial hyphae in surface-grown cultures. At least 23 genes are required for Red biosynthesis (Coco *et al.*, 1991; Feitelson *et al.*, 1985; Rudd & Hopwood, 1980); See http://www.sanger.ac.uk/Projects/S_coelicolor/, all of which are clustered together on the chromosome along with *redD*, encoding the pathway-specific regulator of the *red*

gene cluster (Bibb, 1996). RedD shows 33% identity to ActII-ORF4 (Bibb, 1996), and like ActII-ORF4, it belongs to the SARP family of transcriptional regulators. Also like ActII-ORF4, RedD is thought to function as a transcriptional activator, since expression of some *red* genes was abolished in a *redD* mutant (Feitelson *et al.*, 1985), and introduction of extra cloned copies of *redD* resulted in overproduction of Red in *S. coelicolor* (Narva & Feitelson, 1990). In addition, the expression of *redD* was found to significantly increase in the transition phase from exponential to stationary phase in liquid culture, and this increase was followed by an increase in expression of a *red* biosynthetic gene and the appearance of the Red antibiotic (Takano *et al.*, 1992). Since the presence of *redD* on a multicopy plasmid resulted in the premature production of Red, it was suggested that the growth-phase dependent production of Red depends on the expression of the *redD* gene (Takano *et al.*, 1992).

The pathway-specific regulation of the *red* gene cluster differs from that of the *act* gene cluster in that it also involves a second pathway-specific regulatory protein, referred to as RedZ. Deletion of the *redZ* gene, which is located within the *red* gene cluster, was found to abolish Red production without affecting Act production, and resulted in a severe reduction in the transcription of *redD*, suggesting that RedZ functions to activate either directly or indirectly, the expression of *redD* (White & Bibb, 1997). Sequence analysis of the *redZ* protein product indicated that it shows similarity to response regulator proteins found in a variety of bacteria (Guthrie *et al.*, 1998; White & Bibb, 1997). Unlike these proteins, however, the activity of RedZ does not appear to be regulated by phosphorylation, as many of the conserved charged residues found in the N-terminal phosphorylation pocket of this family of proteins are replaced with hydrophobic

resides in RedZ, including the aspartate residue which is typically the phosphorylation site (Guthrie *et al.*, 1998). Given the hydrophobic nature of this region of RedZ and given the fact that transcription of *redZ* is negatively autoregulated (White & Bibb, 1997), it has been speculated that RedZ may bind either a small hydrophobic regulator molecule, or another regulatory protein, which in turn would mediate RedZ activity. In addition, it has been demonstrated that the transcription of *redZ* is growth phase-dependent, and that *redZ* contains a *bldA*-dependent TTA codon (Guthrie *et al.*, 1998; White & Bibb, 1997), suggesting that the involvement of RedZ as a second pathway-specific regulator of Red production may allow for the input of additional developmental signals at the levels of transcriptional, translational and (possibly) post-translational regulation of *redZ*.

Methylenomycin A is one of only four antibiotics produced by *Streptomyces* spp. whose genes for biosynthesis, resistance and regulation are located on a plasmid (Wright & Hopwood, 1976b). The other three examples are seen in *S. rochei* with lankacidin and lakamycin production, and in *S. rimosus* with oxytetracycline production (Gravius *et al.*, 1994; Suwa *et al.*, 2000). Also unusual about methylenomycin A is that the pathway-specific regulator of the methylenomycin gene cluster, designated MmyR, is a repressor protein that shows similarity to members of the TetR family of transcriptional repressor proteins (Chater & Bruton, 1985). This is in contrast to ActII-ORF4 and RedD, as well as many other pathway-specific regulators for other antibiotics produced by *Streptomyces* spp., which generally function as transcriptional activator proteins (Champness & Chater, 1994). While less is known about methylenomycin A in comparison to the pigmented antibiotics, a recent study suggested that production of the antibiotic may be triggered by

alanine growth-limiting conditions and/or by acidic pH shock, since exposure to either condition resulted in transcription of at least one of the methylenomycin biosynthetic genes, and in the appearance of methylenomycin A (Hayes *et al.*, 1997). Presumably, the sensing of these environmental signals involves MmyR alone, or in conjunction with other proteins that may affect MmyR binding and repression of the biosynthetic gene cluster.

As with methylenomycin A, the fourth antibiotic produced by *S. coelicolor*, known as calcium-dependent antibiotic (CDA), has not been well characterized. CDA is a cyclic lipopeptide composed of six amino acid residues linked to a six-carbon fatty acid chain (Kempter *et al.*, 1997), and which functions by producing transmembrane channels that conduct monovalent cations in the presence of calcium ions (Lakey *et al.*, 1983). The peptide component is thought to be synthesized non-ribosomally by a peptide synthase (Hopwood *et al.*, 1995), and the recent cloning of genes required for CDA production seems to support this notion (Chong *et al.*, 1998). The CDA gene cluster is located on a 98 kb DNA fragment and consists of a number of genes, including *cdaR*, encoding a putative pathway-specific regulator that is homologous to proteins of the SARP family (Ryding *et al.*, 2002). Interestingly, at least five other putative transcriptional regulator genes are also associated with the *cda* gene cluster, which may or may not function together with CdaR to regulate CDA production (Ryding *et al.*, 2002).

In addition to pathway-specific regulatory genes, a number of genes in *S. coelicolor* have been found to function in the global regulation of antibiotic production. One example is the *absA* locus, which was identified in a screen for mutants that were

defective in the production of Act and Red (and were subsequently found to also be defective in Mmy and CDA production) but were still able to undergo morphological differentiation (Adamidis *et al.*, 1990). The inability to synthesize Act and Red in these mutants was shown to result from a severe reduction in transcription of the pathway-specific regulators *actII*-ORF4 and *redD* (Aceti & Champness, 1998). On the other hand, the deficiency in CDA production was due to loss in expression of *cda* biosynthetic genes without any effect on the expression of *CdaR* or any other putative transcriptional regulator in the *cda* gene cluster (Ryding *et al.*, 2002). Interestingly the *absA* locus, like *cdaR*, is located within the *cda* gene cluster (http://www.sanger.ac.uk/Projects/S_coelicolor/), which has led to the suggestion that *absA* may function primarily in the regulation of the *cda* biosynthetic genes, while the regulation of Act and Red production via *actII*-ORF4 and *redD* may be by a more indirect mechanism (Ryding *et al.*, 2002).

Sequence analysis of *absA* revealed that it is composed of two genes, *absA1* and *absA2*, encoding the histidine sensor kinase and the cognate DNA binding response regulator protein, respectively, of a two-component signal transduction system (Brian *et al.*, 1996). The point mutations that resulted in the antibiotic-deficient phenotype were located within the transmitter domain of AbsA1, which normally harbors the kinase and phosphatase activities of histidine kinases (Anderson *et al.*, 1999; Anderson *et al.*, 2001). An unexpected finding, however, was that disruption of *absA1* or *absA2* resulted in premature and enhanced production of Act, Red and CDA, suggesting that AbsA1 and AbsA2 actually function to negatively regulate antibiotic production (Anderson *et al.*, 2001; Brian *et al.*, 1996). This negative regulation most likely involves the

phosphorylation of AbsA2 by AbsA1, since mutation of residues in AbsA2 and AbsA1 that are predicted to be required for phosphorylation of AbsA2, and for the kinase activity of AbsA1, also caused overproduction of antibiotics (Anderson *et al.*, 2001). If AbsA1/A2 do in-fact function as negative regulators, then what is the explanation for the observed antibiotic-deficient phenotype of the AbsA1 point mutants? One proposal is that the point mutations might abolish the phosphatase activity of AbsA1, or they might enhance the kinase activity, such that AbsA2 is always phosphorylated, and antibiotic production is always repressed (Anderson *et al.*, 2001).

Another gene in *S. coelicolor* that is involved in the global regulation of antibiotic production, but not of morphological differentiation, is *afsR*. *afsR* was first identified as a gene that could stimulate the production of Act and Red in *Streptomyces lividans*, where the biosynthetic genes for these antibiotics are normally poorly expressed (Horinouchi *et al.*, 1983). Subsequent studies indicated that the disruption of *afsR* results in significant, but not complete, loss of Act, Red and CDA production (Umeyama *et al.*, 2002), confirming the function of AfsR as a global activator of antibiotic production. The *afsR* gene is located next to other genes that have been implicated in secondary metabolite production, including *afsK*, *kbpA*, and *afsS* (Umeyama *et al.*, 2002). *afsK* encodes a membrane-associated protein showing similarity to eukaryotic Ser/Thr kinases and which autophosphorylates on serine and threonine residues (Matsumoto *et al.*, 1994). *In vitro* phosphorylation assays demonstrate that AfsK can phosphorylate AfsR, however this phosphorylation is inhibited by the binding of the *kbpA* gene product (KbpA, AfsK binding protein) to AfsK, which prevents autophosphorylation of AfsK (Umeyama & Horinouchi, 2001). Since overexpression of KbpA in *S. coelicolor* decreases the

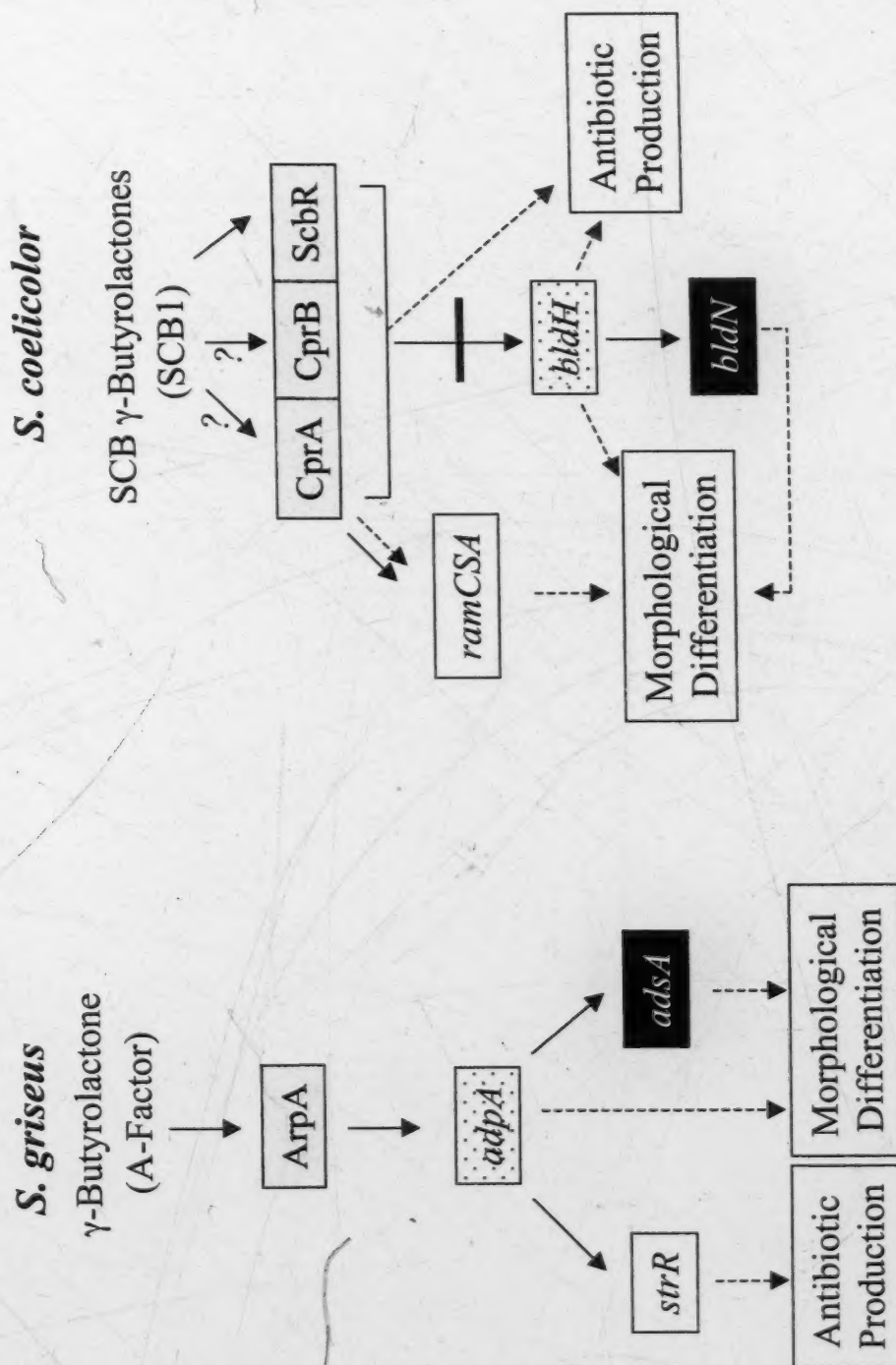
production of Act, and disruption of *kbpA* results in Act overproduction, this suggests that phosphorylation of AfsR is required to activate antibiotic production, and that KbpA may function in a negative feedback system to prevent the continual stimulation of antibiotic production (Umeyama & Horinouchi, 2001). The phosphorylation of AfsR has recently been shown to depend on two other Ser/Thr kinases [PkaG and SCD10.09; (Umeyama *et al.*, 2002)] in addition to AfsK, which may, or may not also be regulated by KbpA.

The N-terminal region of the *afsR* gene product shows 33% identity to ActII-ORF4 and RedD, suggesting that AfsR functions as a DNA-binding transcriptional activator protein (Bibb, 1996). One possible target of AfsR is the *afsS* gene located downstream of *afsR*, as transcription of *afsS* is not detected in an *afsR* mutant (Lee *et al.*, 2002). *afsS* encodes a 63 amino acid protein of unknown function that contains three repeats of Thr-X(2)-Aps-His-Met-X(2)-Pro-Ala, where X is a non-conserved amino acid (Lee *et al.*, 2002). Since overexpression of AfsS activates transcription of *actII*-ORF4, and AfsS is also thought to activate the transcription of *redD* (Floriano & Bibb, 1996; Umeyama *et al.*, 2002), it has been speculated that the repeat sequences allow interaction of AfsS with another protein, which in turn leads to the direct, or indirect activation of the pathway-specific regulatory genes. Recent studies have shown that purified AfsR is able to bind to and stimulate transcription from the *afsS* promoter, and that this binding activity of AfsR is enhanced by phosphorylation (Lee *et al.*, 2002). It is assumed, however, that phosphorylated AfsR also activates other genes involved in secondary metabolite production, as an *afsR* null mutant is almost completely defective in Act

production whereas an *afsS* null mutant still produces moderate levels of antibiotics (Lee *et al.*, 2002).

The nature of the signal(s) that are detected by the *absA* and/or *afs* regulatory systems and which lead to secondary metabolite production are currently unknown, although it has been suggested that both systems respond to one or more external stimuli (Brian *et al.*, 1996; Umeyama *et al.*, 2002). A group of small, diffusible signaling molecules which have been implicated in triggering antibiotic production at very low concentrations are the γ -butyrolactones. The best characterized γ -butyrolactone, A-factor, is necessary for both morphological differentiation and for the production of the antibiotic streptomycin in *Streptomyces griseus* (Horinouchi & Beppu, 1994). *S. coelicolor* produces at least four of these signaling molecules during early stationary phase that can elicit precocious antibiotic production in the wild type strain, and one of these molecules, SCB1, is structurally-related to A-factor (Takano *et al.*, 2000). Unlike A-factor, however, in *S. coelicolor* these molecules do not seem to be involved in the formation of aerial hyphae (Chater & Horinouchi, 2003). The effect of A-factor on morphological and physiological differentiation *S. griseus* is mediated through *adpA*, the transcription of which is repressed by the A-factor receptor protein ArpA during vegetative growth, when A-factor levels are low [(Chater & Horinouchi, 2003; Ohnishi *et al.*, 1999); see Figure 1.4.1]. When a critical concentration of A-factor is reached at a later stage of growth, A-factor binds to ArpA and causes it to dissociate from the *adpA* promoter, thereby allowing expression of *adpA* (Ohnishi *et al.*, 1999; Onaka & Horinouchi, 1997). AdpA is an AraC-like protein that activates transcription of *strR*, the pathway-specific activator for streptomycin biosynthesis (Vujaklija *et al.*, 1993; Vujaklija

Figure 1.4.1 Comparison of orthologous genes and factors involved in the onset of morphological and physiological differentiation in *S. griseus* and *S. coelicolor*. The *S. griseus* A-factor cascade is initiated by the binding of the A-factor γ -butyrolactone to the ArpA receptor protein, which causes the repression of *adpA* by ArpA to be relieved. AdpA then activates transcription of the *strR* gene encoding the pathway-specific regulator for streptomycin production, and of *adsA*, encoding an ECF-sigma factor involved in morphological differentiation, as well as other genes required for morphological differentiation. The *S. coelicolor* ArpA-like proteins, CprA, CprB and ScbR, are known or predicted to interact with A-factor-like signaling molecules (e.g. SCB1), which may result in expression of genes required for morphological (e.g. the *ram* genes in the case of CprA) and/or physiological differentiation. In this case, however, expression of the *adpA* orthologue *bldH* is not dependent on the SCB γ -butyrolactones, and therefore may not be dependent on the ArpA-like proteins. The *adsA* orthologue (*bldN*) which like *AdsA* is required for morphological differentiation, is dependent on *bldH*, and *bldH* is additionally required for antibiotic production and possibly for expression of other genes involved in aerial hyphae formation. Solid arrows represent a known or predicted direct regulatory dependence, whereas broken arrows represent a known or predicted indirect dependence that likely involves multiple steps. Arrows with bars indicate a probable lack of dependence, whereas a solid arrow with (?) indicates a predicted direct dependence. ArpA/CprA,B,ScbR are indicated in gray, whereas AdpA/BldH are indicated as dotted boxes, and AdsA/BldN are indicated as black boxes.



et al., 1991), and of *adsA*, which encodes an ECF-sigma factor that is required for morphological differentiation (Yamazaki *et al.*, 2000). Interestingly, the *S. coelicolor* orthologues of *adpA* and *adsA* are *bldH* and *bldN*, respectively, which is consistent with the observed dependence of *bldN* expression on *bldH* (see Section 1.3). However, the transcription of *bldH* in *S. coelicolor* does not appear to be dependent on the SCB γ -butyrolactones, in contrast to regulation of *adpA* in *S. griseus* (Chater & Horinouchi, 2003).

At least four ArpA-like proteins are also encoded in the *S. coelicolor* genome, including *scbR*, which is divergently transcribed from a gene (*scbA*) that is required for the synthesis of the SCB γ -butyrolactones (Takano *et al.*, 2001). ScbR negatively regulates transcription from its own promoter, which in turn is relieved by the binding of SCB1 to ScbR, and there is evidence that ScbR also functions as a transcriptional activator protein. The role of ScbR and its cognate γ -butyrolactone in the regulation of secondary metabolite production, however, appears to be much more complex than observed for ArpA/A-factor in *S. griseus* (Takano *et al.*, 2001). Two other ArpA-like genes, *cprA* and *cprB*, encode proteins which unlike ScbR, are involved in the regulation of both antibiotic production and morphogenesis. Disruption of *cprA* results in a reduction in antibiotic production and a delay in morphological differentiation, whereas disruption of *cprB* causes precocious overproduction of antibiotics, and accelerated sporulation, suggesting that CprA and CprB function as positive and negative regulators of differentiation, respectively (Onaka *et al.*, 1998). As previously discussed, the expression of *ramC* which is involved in aerial hyphae formation depends directly or indirectly on *cprA*, which partially accounts for the effect of CprA on morphological

differentiation. However, if CprA/B do interact with one or more of the SCB γ -butyrolactones as expected, then it is unclear as to why γ -butyrolactone production appears not to be required for morphological differentiation in *S. coelicolor*.

1.5 Alternate Sigma Factors and the Regulation of Gene Expression

While the growth-phase dependent regulation of developmental genes in *Streptomyces* spp. involves a variety of transcriptional activator and/or repressor proteins, it is apparent that these organisms as well as other organisms employ an auxiliary mechanism of gene regulation that depends on alternative sigma factors. The sigma factor subunit of RNA polymerase holoenzyme directs the enzyme complex to transcribe genes that have a particular promoter sequence (Wosten, 1998). The ability to produce multiple sigma factor subunits allows an organism to specify the transcription of particular sets of genes based on the sigma factor that is associated with the core RNA polymerase. An example of this is seen in the formation of endospores in *B. subtilis*, which involves the creation of two different cell types, one designated the forespore (prespore) which is destined to become the endospore, and the other is called the mother cell which eventually lyses in order to release the mature spore. This process is controlled by a complex regulatory network that involves the sequential activation of four alternative sigma factors (σ^F , σ^E , σ^K , σ^G), each of which governs the temporal and spatial expression of distinct sets of genes that are necessary for the proper development of the spore (Kroos *et al.*, 1999). In addition, alternative sigma factors in many organisms are known to play a role in the expression of flagellar and chemotactic genes, as well as genes

required for survival in response to heat shock and other stresses, and genes which are expressed in response to extracytoplasmic signals (Wosten, 1998).

The existence of multiple sigma factors in an organism necessitates the presence of control mechanisms to regulate their activity. The use of transcriptional activator/repressor proteins to regulate the expression of the sigma factor genes themselves is one way in which this has been accomplished. As well, sigma factor gene expression may involve a cascade of different sigma factor proteins, where transcription of one sigma factor gene is dependent upon the presence of another alternative sigma factor in the cell. For example in sporulating *B. subtilis*, transcription of the gene encoding the forespore-specific σ^G requires another sporulation-specific sigma factor, σ^F , and expression of the gene for the mother cell-specific σ^K requires the presence of active σ^E (Kroos *et al.*, 1999). An additional level of sigma factor control may be implemented through the use of various mechanisms for the post-translational regulation of sigma factor activity. Such mechanisms may entail the controlled degradation of a sigma factor protein, as in the case of the heat-shock-specific sigma factor σ^H of *E. coli* (Yura & Nakahigashi, 1999). Alternatively, a sigma factor may be synthesized initially as an inactive pro-protein precursor, with the subsequent proteolytic processing of this precursor leading to activation of the sigma factor. Examples of this have been seen with the activation of the above mentioned σ^E and σ^K sigma factors of *B. subtilis* (Kroos *et al.*, 1999), and a similar mechanism of sigma factor regulation has been proposed for the BldN sporulation-specific sigma factor of *S. coelicolor* (Bibb & Buttner, 2003). In addition, sigma factor activity may be regulated by interaction of the sigma factor with an

anti-sigma factor protein, which typically prevents the association of the sigma factor with core RNA polymerase (Brown & Hughes, 1995).

Many ECF-sigma factors which control the expression of genes in response to various stresses, as well as genes involved in carotenoid and alginate biosynthesis, are regulated in this manner by anti-sigma factor proteins that are often located in the cytoplasmic membrane (Helmann, 1999). In these cases, the anti-sigma factor responds to external signals transmitted by interaction with extracytoplasmic proteins or small molecules, which results in release of the ECF-sigma factor to allow gene expression. A unique mechanism of sigma factor/anti-sigma factor regulation has been observed in *Salmonella typhimurium* with the flagellar-specific sigma factor σ^{FliA} . This sigma factor is sequestered by the FlgM anti-sigma factor during the early stages of flagellar synthesis, and once the hook and basal body structure is assembled, FlgM is exported from the cell by the flagellar export machinery, thereby freeing σ^{FliA} and allowing it to direct the transcription of the late flagellar genes (Helmann, 1999).

The post-translational regulation of a sigma factor by an anti-sigma factor can also occur using a partner-switching mechanism, in which the anti-sigma factor protein binds to either the sigma factor, or to a protein known as an anti-anti-sigma factor (also called an anti-sigma factor antagonist). The latter interaction typically leads to the accumulation of uninhibited sigma factor protein in the cell, which then allows for the expression of genes under control of that particular sigma factor. One of the best characterized regulatory systems involving these types of proteins is in *B. subtilis* with the regulation of the sporulation-specific σ^{F} sigma factor. σ^{F} is the first compartment-specific sigma factor to become active during sporulation, and it is required for the

expression of genes in the prespore compartment, such as that encoding σ^G (Partridge *et al.*, 1991). Although σ^F is synthesized shortly after the onset of sporulation and prior to the formation of the asymmetric sporulation septum (Gholamhoseinian & Piggot, 1989; Min *et al.*, 1993), the activity of σ^F is confined to the prespore compartment (Margolis *et al.*, 1991). This compartment-specific regulation of σ^F activity is mediated by three proteins, SpoIIAA, SpoIIAB, and SpoIIIE (Errington, 2001; Stragier & Losick, 1996), and two of these proteins, SpoIIAA and SpoIIAB, are encoded along with σ^F in a tricistronic operon (Fort & Piggot, 1984). SpoIIAB is an anti-sigma factor that binds to σ^F in the presence of ATP and holds it in an inactive complex (Duncan & Losick, 1993; Duncan *et al.*, 1996; Min *et al.*, 1993). SpoIIAA is an anti-anti-sigma factor that forms a complex with SpoIIAB in the presence of ADP, thereby freeing σ^F and allowing it to direct transcription (Alper *et al.*, 1994; Duncan *et al.*, 1996; Ho *et al.*, 2003). Interestingly, SpoIIAA is in turn negatively regulated by SpoIIAB, which also functions as a protein kinase that phosphorylates SpoIIAA on a serine residue (Ser58) and therefore prevents SpoIIAA from interacting with SpoIIAB (Alper *et al.*, 1994; Diederich *et al.*, 1994; Magnin *et al.*, 1996; Min *et al.*, 1993; Najafi *et al.*, 1995). Finally, SpoIIIE functions as a septum associated phosphatase that specifically dephosphorylates SpoIIAA in the prespore compartment, and therefore converts it back to the active form (Arigoni *et al.*, 1996; Duncan *et al.*, 1995; Feucht *et al.*, 1996).

A working model for how the compartment-specific regulation of σ^F is carried out is shown in Figure 1.5.1. Prior to septum formation, SpoIIAA is phosphorylated by SpoIIAB using ATP as the phosphoryl group donor, and since SpoIIAA-P cannot interact with SpoIIAB, this allows SpoIIAB to bind σ^F and inhibit its activity. Once the

Figure 1.5.1 Regulation of the *Bacillus subtilis* sporulation-specific σ^F sigma factor.

(A) Prior to the formation of the asymmetric sporulation septum, σ^F is bound to and inhibited by the SpoIIAB anti-sigma factor in a complex that includes ATP. SpoIIAB also possesses kinase activity towards the SpoIIAA anti-anti-sigma factor, which results in inactivation of that protein as well. (B) Once septum formation occurs, SpoIIAA is rapidly dephosphorylated in the prespore compartment by the septum-associated SpoIIE phosphatase, and unphosphorylated SpoIIAA then induces the release of σ^F from the SpoIIAB-ATP- σ^F complex. The SpoIIAB-ADP complex, which presumably results from phosphorylation of SpoIIAA during the induced release of σ^F , is a long-lived complex that cannot function as a kinase, but is able to interact with unphosphorylated SpoIIAA generated by SpoIIE. This interaction sequesters SpoIIAB, and allows σ^F to direct gene transcription. Adapted from Helmann (1999) and Ho *et al.* (2003). AA, SpoIIAA; AB, SpoIIAB; P, phosphate group.

asymmetric septum is formed, however, both SpoIIAB and SpoIIE are active in the prespore compartment, and as a result SpoIIAA is cycled between the phosphorylated and unphosphorylated forms (Magnin *et al.*, 1997). Since the rate of dephosphorylation of SpoIIAA-P exceeds that of the SpoIIAB kinase activity (Lucet *et al.*, 1999), the unphosphorylated form of SpoIIAA accumulates, and this form of SpoIIAA is then able to induce the release of σ^F from the SpoIIAB- σ^F complex (Duncan *et al.*, 1996; Ho *et al.*, 2003). In addition, phosphorylation of SpoIIAA by SpoIIAB [which may also occur during the induced release of σ^F ; (Duncan *et al.*, 1996); see Figure 1.5.1] results in release of a long-lived SpoIIAB-ADP molecule that is enzymatically inactive, but can still form a complex with unphosphorylated SpoIIAA generated by SpoIIE (Lee *et al.*, 2000). Since there is just enough SpoIIAB present to inhibit σ^F (Magnin *et al.*, 1997), the sequestering of even a small amount of SpoIIAB in complexes with SpoIIAA will result in the presence of uninhibited σ^F molecules. As well, it has recently been shown that SpoIIAB is subject to proteolysis by a ClpCP protease, which may serve as an additional mechanism for the prespore-specific activation of σ^F (Pan *et al.*, 2001).

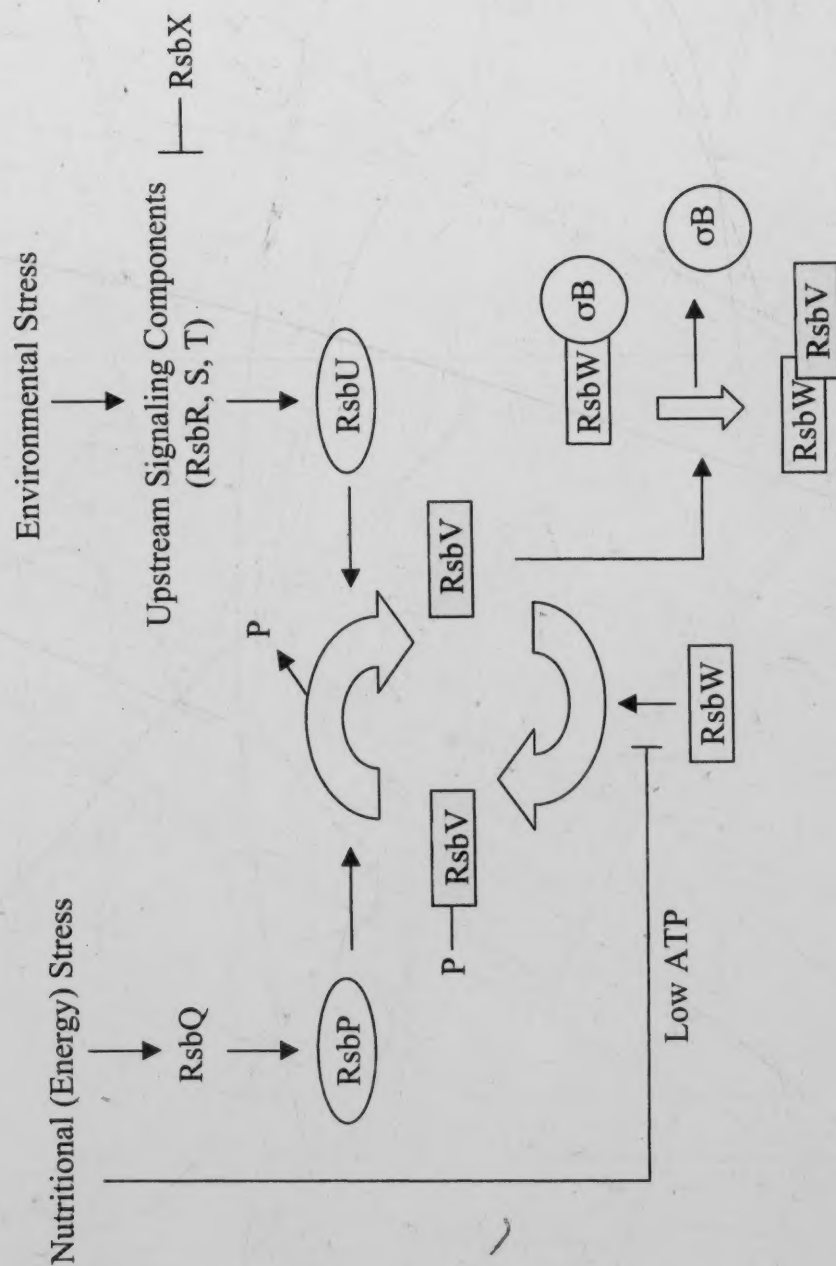
A second sigma factor of *B. subtilis* that is also known to be regulated by anti-sigma factor and anti-anti-sigma factor proteins is the general stress response sigma factor σ^B . This sigma factor is responsible for the transcription of a subset of genes in response to nutritional stress (i.e. glucose, oxygen, phosphate deprivation), as well as in response to different environmental stresses such as ethanol shock, osmotic shock, acid shock and heat shock (Benson & Haldenwang, 1993c; Boylan *et al.*, 1993a; Boylan *et al.*, 1993b; Voelker *et al.*, 1995b; Voelker *et al.*, 1994). Two pathways for the activation of σ^B have been identified, one which is specific for environmental stresses and involves the

RsbU, S, T, R, and X regulatory proteins (rsb = Regulator of SigmaB) (Akbar *et al.*, 1997; Gaidenko *et al.*, 1999; Kang *et al.*, 1996; Smirnova *et al.*, 1998; Voelker *et al.*, 1995a; Voelker *et al.*, 1995b; Yang *et al.*, 1996), and the other which is specific for nutritional (energy) stress and involves RsbP and RsbQ (Brody *et al.*, 2001; Vijay *et al.*, 2000). These two pathways, as shown in Figure 1.5.2, converge upon the common principle regulators RsbW, which functions as an anti-sigma factor of σ^B (Alper *et al.*, 1996; Benson & Haldenwang, 1993a; Benson & Haldenwang, 1993b), and RsbV, an anti-anti-sigma factor that counteracts the inhibition of σ^B by RsbW (Alper *et al.*, 1996; Dufour & Haldenwang, 1994). All of the Rsb regulatory proteins are encoded along with σ^B in an operon with the exception of RsbQ and P, which are encoded elsewhere on the chromosome as a separate operon (Brody *et al.*, 2001; Kalman *et al.*, 1990; Wise & Price, 1995).

The phosphorylation state of the RsbV anti-anti-sigma factor, which is controlled by a balance between the kinase activity of RsbW and the phosphatase activities of RsbU and RsbP (Dufour & Haldenwang, 1994; Vijay *et al.*, 2000; Voelker *et al.*, 1996; Yang *et al.*, 1996), determines whether RsbW will bind to σ^B or to RsbV, and therefore determines whether σ^B will be active or not. In the absence of stress, the phosphorylated form of RsbV (RsbV-P) is presumed to be predominant, and σ^B is held in an inactive complex with RsbW. On the other hand, when *B. subtilis* enters stationary phase and experiences nutritional stress, RsbV accumulates as a result of the inefficient phosphorylation of RsbV by RsbW under conditions of low ATP (Alper *et al.*, 1996; Delumeau *et al.*, 2002; Voelker *et al.*, 1995b), and because of the activity of the RsbV-specific phosphatase RsbP, which in turn may be activated by the RsbQ protein by an

Figure 1.5.2 Model for the regulation of the stress response sigma factor σ^B of *Bacillus subtilis*. In the absence of stress, the RsbV anti-anti-sigma factor (gray box) is phosphorylated by the RsbW anti-sigma factor, and is therefore inactivated. RsbW is then able to form a complex with σ^B and prevent its association with core RNA polymerase. Under conditions of nutritional (energy) stress, the RsbP phosphatase in concert with RsbQ dephosphorylates RsbV, and phosphorylation of RsbV by RsbW is also thought to be inefficient due to lower levels of ATP under these conditions. In response to environmental stress, a signal transduction system involving RsbS, T, and R activates the RsbU phosphatase, which then dephosphorylates RsbV. In both cases, this leads to the accumulation of active RsbV, which then binds to RsbW, holding it in an inactive complex, and allowing σ^B to direct transcription of the σ^B regulon. The RsbX protein is thought to limit the degree of the stress response and to restore σ^B activity to pre-stress levels following induction.

5



unknown mechanism. In the case of environmental stresses, activation of RsbV occurs through the RsbU phosphatase, which is regulated by an elegant system involving the RsbR, S, T, and X proteins. It is thought that these proteins function in the transmission of environmental signals to RsbU, which then leads to the accumulation of active RsbV and expression of the σ^B regulon. In the case of RsbX, which is a negative regulator of the stress activation pathway, this protein may serve to limit the degree of the stress response and to restore σ^B activity to pre-stress levels following induction (Scott *et al.*, 2000; Smirnova *et al.*, 1998; Voelker *et al.*, 1997).

The existence of multiple sigma factors in *S. coelicolor* was first demonstrated by Westpheling *et al.* (1985), who identified two distinct RNA polymerase holoenzymes that could each recognize one of two well-characterized promoter sequences from *B. subtilis* in *in vitro* transcription assays. An extension of this idea of RNA polymerase heterogeneity was later revealed by the discovery of four homologs of the vegetative σ^{70} (RpoD) sigma factor of *E. coli*, which were named HrdA, HrdB, HrdC, and HrdD (Hrd = Homologs of rpoD) (Tanaka *et al.*, 1988). Of these four sigma factors, however, only HrdB is thought to function as the principle vegetative sigma factor, since it was found to be the most abundant sigma factor associated with core RNA polymerase, and disruption of *hrdB* was lethal to the organism, whereas disruption of the other three *hrd* genes had no phenotypic effects (Brown *et al.*, 1992; Buttner & Lewis, 1992; Buttner *et al.*, 1990). Soon, it became apparent that many streptomycete genes are expressed from more than one promoter, and that promoter sequences display a high degree of heterogeneity (Bibb, 1996), which in turn might specify transcription by different sigma factor subunits. For example the *dagA* gene, which encodes an extracellular agarase enzyme, was found to be

transcribed from four different promoters, three of which were shown to be recognized by separate RNAP holoenzymes (Brown *et al.*, 1992; Buttner *et al.*, 1988). More recently, it has been demonstrated that distinct sets of promoters are preferentially recognized by either exponential or stationary phase RNAP, and that this growth-phase-specific promoter selectivity is determined by the associated sigma factor subunits (Kang *et al.*, 1997). Indeed, the importance of alternative sigma factors in the regulation of gene expression in *Streptomyces* is illustrated by the existence of 65 sigma factor genes in the *S. coelicolor* genome, the largest number of sigma factors encoded by an organism to date (Bentley *et al.*, 2002). Of these sigma factors, 45 belong to the ECF family of sigma factors, while nine appear to be homologs of the *B. subtilis* general stress response sigma factor σ^B .

The recent characterization of a number of alternative sigma factors in *S. coelicolor* has implicated these proteins as key regulators of both morphological differentiation and of various stress response systems. As previously mentioned, the WhiG and SigF sigma factors are involved in the early and late stages of sporulation, respectively, while the *bldN*-encoded ECF sigma factor appears to be required for the erection of aerial hyphae. Sigma factors which function in the adaptation to stress include the SigE ECF-sigma factor, which is necessary for normal cell wall integrity (Lonetto *et al.*, 1994; Paget *et al.*, 1999b), and SigR, which induces the thioredoxin system as well as other genes involved in thiol metabolism in response to disulfide stress (Paget *et al.*, 1998; Paget *et al.*, 2001a). The regulation of *sigE* is controlled by a two-component signal transduction system that is thought to respond to the accumulation of intermediates in peptidoglycan synthesis or degradation (Hong *et al.*, 2002; Paget *et al.*,

1999a). SigR, on the other hand, is post-translationally regulated by an upstream redox-sensitive anti-sigma factor, RsrA, which binds SigR in the absence of disulfide stress, and releases SigR under oxidizing conditions that induce intramolecular disulfide bond formation in RsrA itself (Kang *et al.*, 1999; Li *et al.*, 2002). Interestingly, an *rsrA* null mutant is unable to sporulate, however a *sigR-rsrA* double mutant could undergo normal development, suggesting that the increase in free SigR may either outcompete a sporulation-specific sigma factor (i.e. WhiG), or that the expression of one or more SigR target genes in the absence of disulfide stress inhibits sporulation (Paget *et al.*, 2001b). A similar situation has been observed with the *S. coelicolor* *rsuA-sigU* operon, which encodes a putative anti-sigma factor and a downstream sigma factor, the function of which is unknown (Gehring *et al.*, 2001). In this case, however, the *rsuA* null mutant has a bald phenotype and shows a severe delay in the production of the blue-pigmented antibiotic actinorhodin, suggesting that the presence of free SigU affects an earlier stage of development compared to SigR.

Of the nine *B. subtilis* σ^B homologs found in *S. coelicolor*, four of these, SigI, SigJ, SigB and SigH, are known to be induced in response to osmotic shock (Cho *et al.*, 2001; Kormanec *et al.*, 2000; Viollier *et al.*, 2003b). In the case of SigB, a constructed null mutant was found to be sensitive to hyperosmolarity, which suggests that SigB is required for osmoprotection in *S. coelicolor* (Cho *et al.*, 2001). SigH, which is expressed from four different promoters, is also induced in response to heat and ethanol shock, whereas expression of SigI, J and B is not (Kormanec *et al.*, 2000; Viollier *et al.*, 2003b). This is in contrast to the situation in *B. subtilis*, where σ^B is activated by a variety of environmental stresses, and suggests that the stress response systems in *S. coelicolor* (i.e.

heat, ethanol, osmotic) are able to function independently of one another. The regulation of both SigB and SigH is thought to involve anti-sigma factor proteins that are encoded next to the respective sigma factor genes (Cho *et al.*, 2001; Sevcikova & Kormanec, 2002; Viollier *et al.*, 2003a). As well, it has recently been shown that *sigH* is expressed as three different translational products, two of which are subjected to proteolytic processing in the later stages of development, while the other is found to accumulate as development proceeds (Viollier *et al.*, 2003a). This is in addition to the transcriptional regulation of the various *sigH* promoters, one which is dependent on SigH itself, and another which is developmentally regulated by the BldD transcriptional regulator (Kelemen *et al.*, 2001; Sevcikova *et al.*, 2001). As previously mentioned, the dependence of SigH expression on BldD offers evidence for a link between stress and development. This link has been further substantiated by recent studies which demonstrated that the expression of catalase B, which is required for osmoprotection and proper differentiation of *S. coelicolor*, is directly dependent on SigB (Cho *et al.*, 2000; Cho *et al.*, 2001).

1.6 Thesis Objectives

The post-translational regulation of alternative sigma factors in *S. coelicolor* by anti-sigma factor proteins has been well documented, however the involvement of anti-anti-sigma factors has not been studied at all. As previously mentioned, one of the *bld* gene global regulators, *bldG*, required for both morphological and physiological differentiation, encodes a putative anti-anti-sigma factor, which suggests that this protein is involved in the regulation of one or more sigma factors that are implicated in these differentiation processes. The objective of this study, therefore, was to characterize *bldG*

in terms of its role in the regulation of differentiation in *S. coelicolor*. This entailed mutational studies of *bldG* and of a downstream open reading frame (designated *orf3*), which was found to encode a protein showing similarity to anti-sigma factor proteins. As well, transcriptional studies were performed to determine the timing of expression of *bldG* during development and to determine whether *bldG* and the downstream open reading frame are co-transcribed as a polycistronic transcript. Since the BldG homologs SpoIIAA and RsbV of *B. subtilis* are both regulated by phosphorylation, experiments were carried out to investigate whether BldG is also post-translationally modified by phosphorylation. Binding studies using purified BldG and ORF3 proteins were also performed to examine whether the two proteins function as binding partners. Finally, the lack of a sigma factor gene in the vicinity of the *bldG* locus prompted attempts to identify targets of the sigma factor proposed to be regulated by BldG, in hopes of identifying the target sigma factor itself, and its direct function in *S. coelicolor* development.

Chapter 2:
Materials and Methods

2. Materials and Methods

2.1 Bacterial Strains, Plasmids and Growth Conditions

2.1.1 *Streptomyces* and *Escherichia coli* strains

Escherichia coli strains used in this study are listed in Table 2.1. *Streptomyces* strains used are listed in Table 2.2.

2.1.2 Plasmid vectors

Cloning vectors used in this study are listed in Table 2.3. Recombinant vectors that were constructed and used are listed in Table 2.4.

2.1.3 Propagation and maintenance of *E. coli* strains

E. coli strains were grown at 30 or 37 °C, where indicated, in LB liquid medium (1% w/v tryptone; 0.5% w/v yeast extract; 1% w/v NaCl) (Sambrook *et al.*, 1989), L-broth (Kieser *et al.*, 2000) containing 0.2% glucose, or SOB medium (Sambrook *et al.*, 1989) on a rotating rack, or on LB solid medium containing 1.5% w/v agar. When necessary, the medium was supplemented with ampicillin (100 µg/ml, Sigma), apramycin (50 µg/ml, Provel), kanamycin (30 µg/ml, Sigma), chloramphenicol (25 µg/ml, Aldrich), tetracycline (10-12.5 µg/ml, Sigma), or spectinomycin (100 µg/ml, Sigma). Strains were maintained at -80 °C as frozen stocks in 20% v/v glycerol.

Table 2.1: *Escherichia coli* strains used in this study

<i>E. coli</i> strain	Genotype	Reference or Source
DH5 α	F, ϕ 80lacZAM15 Δ (lacZYA-argF)U169, deoR, recA1, hsdR17(<i>r_K</i> , <i>m_K</i> -) <i>phoA</i> , <i>supE44</i> , λ -, <i>thi</i> -1, <i>gyrA96</i> , <i>relA1</i>	(Hanahan, 1983); Gibco BRL
ET12567	F, <i>dam</i> 13::Tn9, <i>dcm</i> 6, <i>hsdR</i> , <i>recF143</i> , <i>zjj202</i> ::TN10, <i>galK2</i> , <i>galT22</i> , <i>ara14</i> , <i>lacY1</i> , <i>xyl5</i> , <i>leuB6</i> , <i>thi1</i> , <i>tonA31</i> , <i>rpsL136</i> , <i>hisG4</i> , <i>tsx78</i> , <i>mll1</i> , <i>glnV44</i>	(MacNeil <i>et al.</i> , 1992); Gift from D. MacNeil, Merck Sharp and Dohme Research Laboratories
ES1301	<i>lacZ53</i> , <i>mutS201</i> ::Tn5, <i>thyA36</i> , <i>rha-5</i> , <i>metB1</i> , <i>deoC</i> , IN(<i>rrnD-rrnE</i>)	(Siegel <i>et al.</i> , 1982)
JM109	<i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> (<i>r_K</i> , <i>m_K</i> +), <i>relA1</i> , <i>supE44</i> , λ -, Δ (<i>lac-proAB</i>), [<i>F</i> *, <i>traD36</i> , <i>proA</i> ⁺ <i>B</i> ⁺ , <i>lacF</i> ⁺ ZAM15]	(Yanisch-Perron <i>et al.</i> , 1985)
BL21(DE3)pLysS	F, <i>ompT</i> , <i>hsdS_B</i> (<i>r_B</i> , <i>m_B</i> -), <i>gal</i> , <i>dcm</i> , (DE3), pLysS (Cm ^R)	Stratagene; Gift from Gary Ritzel, University of Alberta
BW25113	Δ (<i>araD-araB</i>)567, Δ lacZ4787(:: <i>rrnB-4</i>), <i>lacIp-4000</i> (<i>lacIq</i>), λ -, <i>rpoS369</i> (Δ m), <i>rph-1</i> , Δ (<i>rhaD-rhaB</i>)568, <i>hsdR514</i>	(Datsenko & Wanner, 2000); Plant Bioscience Limited, U.K.
One Shot™ TOP10	F, <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>), Φ 80lacZAM15, <i>lacX74</i> , <i>recA1</i> , <i>deoR</i> , <i>araD139</i> , Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (<i>Str^R</i>), <i>endA1</i> , <i>nupG</i>	Invitrogen

Table 2.2: *Streptomyces* strains used in this study

<i>Streptomyces coelicolor</i> A3(2)	Genotype	Reference and/or Source
J1501	<i>hisA1, uraA1, strA1, pgI, SCP1⁻, SCP2⁻</i>	(Chater <i>et al.</i> , 1982); John Innes Institute
M145	prototrophic, <i>SCP1⁻ SCP2⁻ Pgl⁺</i>	(Lomovskaya <i>et al.</i> , 1980); John Innes Institute
M600	<i>SCP1⁻, SCP2⁻</i>	(Chakraburty & Bibb, 1997); Gift from M. Buttner, John Innes Centre
C3b	J1501 derivative with a thiostrepton-resistance cassette inserted into the <i>Bgl</i> II site of <i>bldG</i>	(Bignell <i>et al.</i> , 2000)
IDB	M145 derivative with an in-frame deletion in <i>bldG</i>	This study
C103	<i>bldG</i> point mutant derived from J1501	(Champness, 1988); Gift from W. Champness, Michigan State University
C536	<i>bldG</i> point mutant derived from J1501	Gift from W. Champness, Michigan State University
C107	<i>bldG</i> point mutant derived from J1501	(Champness, 1988); Gift from W. Champness, Michigan State University
C101J	Bald mutant derived from J1501	(Champness, 1988); Gift from W. Champness, Michigan State University

Table 2.3: Plasmids and cloning vectors used in this study

Plasmid	Antibiotic Marker	Relevant Characteristics	Reference and/or Source
<i>E. coli</i> Plasmids, Phagemids and Cosmids			
pBluescript® KS ⁺ , SK ⁺	Ampicillin	High copy number phagemid containing T7 and T3 polymerase promoters.	(Alting-Mees & Short, 1989); Stratagene
pMAL-c2X	Ampicillin	High copy number vector for overexpression of MBP-fusion proteins	New England Biolabs
pCR2.1TOPO	Ampicillin, Kanamycin	High copy number phagemid for rapid cloning of PCR-generated fragments	Invitrogen
pAlter-Ex2	Tetracycline	Phagemid cloning vector for <i>in vitro</i> site-specific mutagenesis	Promega
pET30(a)+ Derivative	Kanamycin	A 453 bp <i>Sph</i> I- <i>Bam</i> HI fragment including part of multi-cloning region is replaced with a 381 bp <i>Sph</i> I- <i>Bam</i> HI fragment from the multi-cloning region of pET19b	Novagen; Gift from S. Jensen, University of Alberta
pUZ8002	Kanamycin	Plasmid encodes transfer functions for mobilization of <i>oriT</i> -containing plasmids from <i>E. coli</i> to <i>Streptomyces</i>	Gift from M. Buttner, John Innes Centre
pIJ773	Ampicillin, Apramycin	pBluescript KS ⁺ derivative containing the apramycin resistance gene [<i>aac</i> (3)/IV] and the <i>oriT</i> of plasmid RP4 in the <i>Eco</i> RV site	(Gust <i>et al.</i> , 2003); Plant Bioscience Limited
pAU64	Ampicillin	pBluescript SK ⁺ derivative containing a 2.5 kb <i>Hind</i> III fragment of <i>S. coelicolor</i> DNA with the entire <i>bldG</i> and partial <i>orf3</i> coding regions	(Bignell <i>et al.</i> , 2000)

Plasmid	Antibiotic Marker	Relevant Characteristics	Reference and/or Source
pAU66	Ampicillin	pBluescript KS+ derivative containing a 2.3 kb <i>Bam</i> HI fragment of <i>S. coelicolor</i> DNA with the entire <i>bldG</i> and <i>orf3</i> coding regions	(Bignell <i>et al.</i> , 2000)
pIJ790	Chloramphenicol	Plasmid carries the genes encoding Red α , Red β , and Red γ of phage λ , the expression of which are under control of the arabinose-inducible <i>ParaBAD</i> promoter. Plasmid also contains the repA101 temperature-sensitive replicon.	(Gust <i>et al.</i> , 2003); Plant Biosciences Limited, U.K.
Cosmid SCH5	Ampicillin, Kanamycin	Supercos-1 derivative containing 40.54 kb of <i>S. coelicolor</i> chromosomal DNA, including the <i>bldG</i> and <i>orf3</i> genes	(Redenbach <i>et al.</i> , 1996); Gift from H. Kieser, John Innes Centre
<i>E. coli-Streptomyces</i> shuttle vectors pSET152	Apramycin	High copy cloning vector in <i>E. coli</i> , integrates into the <i>Streptomyces attB</i> site.	(Bierman <i>et al.</i> , 1992); Northern Regional Research Center, Peoria, Ill.
pSETQ	Spectinomycin	pSET152 derivative in which the apramycin resistance cassette (<i>aac(3)/IV</i>) is replaced with a <i>Sac</i> I fragment containing the spectinomycin resistance gene (<i>aad</i>)	(O'Connor <i>et al.</i> , 2002); Gift From J. Nodwell, McMaster University
pIJ6902	Apramycin	Contains the thiostrepton-inducible promoter <i>ptipA</i> from <i>Streptomyces</i>	Gift from M. Buttner, John Innes Centre
pT06	Spectinomycin	pIJ8860 derivative in which the apramycin resistance cassette (<i>aac(3)/IV</i>) is replaced with the spectinomycin resistance gene (<i>aad</i>).	(O'Connor <i>et al.</i> , 2002); Gift from J. Nodwell, McMaster University

Plasmid	Antibiotic Marker	Relevant Characteristics	Reference or Source
pHIL400	Ampicillin, Thiostrepton	High copy number vector in <i>E. coli</i> , <i>par</i> ⁺ in <i>Streptomyces</i>	(Larson & Hershberger, 1986); Gift from S. Jensen, University of Alberta

Table 2.4: Recombinant plasmids and cosmids constructed and used in this study

Plasmid/ Cosmid	Parent Plasmid/Cosmid	Selective Marker(s)	Insert size	Relevant Characteristics	Use
pAU69	pSET152	Apramycin	917 bp	Vector contains the entire <i>bldG</i> coding region and upstream promoter on an <i>EcoRI</i> - <i>XbaI</i> fragment	Complementation of <i>bldG</i> mutants
pAU308	pMAL-c2X	Ampicillin	347 bp	The <i>bldG</i> coding sequence was cloned as an <i>XmnI</i> - <i>HindIII</i> fragment immediately downstream of the MBP gene sequence; expression is under control of <i>ptac</i> promoter	Overexpression of MBP-BldG in <i>E. coli</i>
pAU310	pCR2.1TOPO	Ampicillin, Kanamycin	527 bp	Vector contains an <i>EcoRI</i> - <i>XbaI</i> PCR product that includes the first 57 bp of <i>bldG</i> and 450 bp of upstream sequence	Construction of <i>bldG</i> in-frame deletion vector
pAU311	pCR2.1TOPO	Ampicillin, Kanamycin	594 bp	Vector contains an <i>XbaI</i> - <i>HindIII</i> PCR product that includes the terminal 33 bp of <i>bldG</i> and 541 bp of downstream sequence	Construction of <i>bldG</i> in-frame deletion vector
pAU312	pCR2.1TOPO	Ampicillin, Kanamycin	528 bp	Vector contains a PCR product that includes the terminal 105 bp of <i>bldG</i> , the <i>bldG-orf3</i> intergenic region and the first 304 bp of <i>orf3</i>	Generation of probe for 3' S1 mapping of <i>bldG</i> monocistronic transcript
pAU314	pHJL400	Ampicillin, Thiostrepton	1097 bp	<i>EcoRI</i> - <i>XbaI</i> fragment from pAU310 and <i>XbaI</i> - <i>HindIII</i> fragment from pAU311 were cloned in-frame into <i>EcoRI</i> - <i>HindIII</i> sites of pHJL400	Introduction of <i>bldG</i> in-frame deletion into <i>Streptomyces</i>
pAU316	pIJ6902	Apramycin	473 bp	<i>bldG</i> is present as an <i>NdeI</i> - <i>XbaI</i> fragment; expression of <i>bldG</i> is under control of <i>ptipA</i> promoter	Overexpression of BldG in <i>Streptomyces</i>

Plasmid/ Cosmid	Parent Plasmid/Cosmid	Selective Marker(s)	Insert size	Cloning Strategy	Use
pAU317	pIJ6902	Apramycin	584 bp	<i>orf3</i> is present as an <i>NdeI-XbaI</i> fragment; expression of <i>orf3</i> is under control of <i>ptipA</i> promoter	Overexpression of ORF3 in <i>Streptomyces</i>
pAU318	pET30(a)+ derivative	Kanamycin	556 bp	<i>orf3</i> was cloned in as an <i>NdeI-EcoRI</i> fragment downstream of 10× histidine tag; expression is under control of the T7 promoter	Overexpression of His ₁₀ -ORF3 in <i>E. coli</i>
pAU319	pAlter-Ex2	Chloramphenicol, Tetracycline	449 bp	Vector contains the entire <i>bldG</i> coding sequence as an <i>NdeI-EcoRI</i> fragment and beginning at <i>bldG</i> start codon	Site-directed mutagenesis of <i>bldG</i>
pAU320	pAlter-Ex2	Chloramphenicol, Tetracycline	449 bp	Same as pAU319, except that there is a single T→G transversion mutation in <i>bldG</i> , and a frameshift mutation in vector to restore chloramphenicol resistance	Site-directed mutagenesis of <i>bldG</i>
pAU321	pMAL-c2X	Ampicillin	349 bp	Vector contains a <i>BamHI-HindIII</i> fragment with the mutant <i>bldG</i> gene from pAU320	Overexpression of MBP-BldGS57A in <i>E. coli</i>
pAU322	pIJ6902	Spectinomycin		pIJ6902 derivative in which the apramycin resistance cassette is replaced with a <i>SacI</i> spectinomycin resistance fragment; the <i>BamHI-BglII</i> fragment from the pIJ6902 multi-cloning region has been removed	Control vector for complementation of <i>orf3</i> deletion mutants
pAU323	pAU317	Spectinomycin	584 bp	Same as pAU322, except that <i>orf3</i> is present as an <i>NdeI-XbaI</i> fragment; expression of <i>orf3</i> is under control of the <i>ptipA</i> promoter	Complementation of <i>orf3</i> deletion mutants

Plasmid/ Cosmid	Parent Plasmid/Cosmid	Selective Markers	Insert size	Relevant Characteristics	Use
pAU324	pSETΩ	Spectinomycin	1097 bp	Vector contains an <i>EcoRI</i> -blunted <i>HindIII</i> fragment from pAU314	Complementation of <i>orf3</i> deletion mutants
pAU325	pAU69	Apramycin	893 bp	<i>EcoRI</i> - <i>XbaI</i> insert contains the <i>bldG</i> SS7A mutant gene and upstream promoter	Complementation <i>bldG</i> deletion mutant
pAU326	Cosmid SCH5	Ampicillin, Kanamycin, Apramycin	41.57 kb	Same as Cosmid SCH5, except that a 354 bp internal region of <i>orf3</i> was replaced with a 1384 bp fragment containing the (<i>aac</i> (3)/IV) apramycin resistance cassette and the <i>oriT</i> of plasmid RP4	Deletion of <i>orf3</i> in <i>S. coelicolor</i>
pAU327	pSET152	Apramycin	1460 bp	<i>EcoRI</i> insert contains the <i>bldG</i> promoter, the <i>bldG</i> coding region, and the <i>orf3</i> coding region	Complementation of <i>bldG</i> 101J mutant

2.1.4 Preparation of *E. coli* glycerol stocks

Glycerol stocks were prepared by mixing equal volumes of an overnight liquid culture with 40% v/v sterile glycerol in screw cap tubes. The stocks were then flash-frozen in a dry ice-ethanol bath, and kept at -80°C .

2.1.5 Propagation and maintenance of *Streptomyces* strains

Streptomyces strains were grown in liquid medium as either 5 ml cultures in universal bottles with a 1 inch spring coil, or as 25-50 ml cultures in 250 ml flasks with a spring coil. Strains were grown where specified in R2YE (Hopwood *et al.*, 1985) broth, Trypticase Soy Broth (TSB, 30 g/L), 2 × YT broth (Sambrook *et al.*, 1989) or NMMP medium (Kieser *et al.*, 2000) containing glucose as the carbon source, and were incubated at 30°C and 200 rpm. Surface cultures of *Streptomyces* were grown where indicated on R2YE agar, Difco nutrient agar (DNA, 23g/L) or MS agar (20 g/L soya flour; 20 g/L mannitol; 20 g/L agar) (Kieser *et al.*, 2000) at 30°C . Where necessary, liquid or solid medium was supplemented with thiostrepton (30-50 $\mu\text{g/ml}$; gift from S. Lucania at Squibb and Sons, Inc., Institute for Medical Research), apramycin (25-50 $\mu\text{g/ml}$) nalidixic acid (25 $\mu\text{g/ml}$, Sigma) or spectinomycin (100-500 $\mu\text{g/ml}$). *Streptomyces* strains were maintained at -20°C in 20% v/v glycerol as either spore or mycelial stocks.

2.1.6 Preparation of *Streptomyces* glycerol stocks

Streptomyces spore and mycelial stocks were prepared by growing strains on cellophane disks on R2YE medium for 4-7 days at 30°C . For spore stocks, the growth was scraped from the plates into sterile universal bottles containing sterile milli-Q water,

and the resulting suspension was placed in a sonication bath for 5-15 minutes to help release the spores from the biomass. After leaving on ice to allow most of the unwanted biomass to settle, the liquid containing the spores was filtered through sterile cotton wool, and the filtrate was centrifuged for 10 minutes at 2900 rpm and 4 °C in an International Centrifuge with a swinging bucket rotor. After pouring off the supernatant, the resulting spores were resuspended in glycerol to give a final concentration of 20%, and were aliquoted into sterile Bijou bottles for storage at -20 °C.

For preparation of *Streptomyces* mycelial stocks, growth from R2YE plates was scraped into ground glass homogenizers containing sterile milli-Q water, and the resulting suspension was homogenized in order to break up the mycelia into small fragments. After centrifugation at 2900 rpm in an International Centrifuge with a swinging bucket rotor, the supernatant was poured off, and the mycelial fragments were resuspended in glycerol to 20% final concentration. The stock was then aliquoted and stored at -20 °C.

2.2 DNA Isolation and Transformation

2.2.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was typically isolated from *E. coli* using the alkali lysis method described by Sambrook *et al.* (1989). Reagent volumes were scaled up accordingly when large-scale preparations were necessary. For high purity DNA preparations, Qiagen tip 100 columns were used as described by the manufacturer.

2.2.2 Preparation of *E. coli* competent cells

Competent cells were prepared by inoculating 2 ml of LB broth + antibiotic (when necessary) with a single *E. coli* colony and growing the culture overnight at 30 or 37 °C. The culture was then diluted 1:100 into fresh LB medium (+ antibiotic), and was incubated with shaking at 30 or 37 °C for about 3-4 hours, or until the OD₆₀₀ was ~0.6. The cells were then pelleted by centrifugation at 2000 rpm and 4 °C in an International Centrifuge with a swinging bucket rotor. When preparing calcium chloride competent cells, the pelleted cells were washed once with 5 ml of 100 mM MgCl₂, then once with 5 ml of 100 mM CaCl₂, and were finally resuspended in 100 mM CaCl₂ and 20% glycerol to a final volume of 1 ml. For preparation of electrocompetent cells, the pelleted cells were washed twice with 10% glycerol and then resuspended in 10% glycerol to a final volume of 1 ml. In both cases, aliquots of 50-100 µl were then used immediately for transformation, or were flash-frozen in a dry ice-ethanol bath and stored at -80 °C.

Electrocompetent cells of *E. coli* BW25113/pIJ790 containing Cosmid SCH5 which were used for PCR-targeted mutagenesis of *orf3* were prepared by inoculating 50 ml of SOB medium containing ampicillin, kanamycin and chloramphenicol with 1% of an overnight culture. L-arabinose (10 mM) was then added to induce expression of the λRed proteins, and the culture was grown at 30 °C until the OD₆₀₀ was ~0.6. Cells were pelleted and washed with 10% glycerol as described above, and were resuspended in 10% glycerol to a final volume of ~100 µl. Aliquots (50 µl) were then used immediately in transformation reactions.

2.2.3 Transformation of *E. coli*

For transformation of calcium chloride competent cells, the cells were thawed on ice, and plasmid DNA (1-5 μ l) was mixed with the cells by pipetting up and down with a pipettor. The mixture was allowed to sit on ice for 30 minutes, and was then heat shocked by incubation at 42 °C for 90 seconds. After sitting on ice for 2 minutes, LB broth was added to a final volume of 1 ml, and the cells were incubated at 37 °C on a rotating rack for 45-60 minutes. For transformation of electrocompetent cells, the cells were thawed on ice and mixed with plasmid DNA as described above. The mixture was then transferred to an ice-cold electroporation cell (0.2 cm), and electroporation was carried out using a Bio-Rad GenePulser II set to 200 Ω , 25 μ F and 2.5 kV. Ice cold LB broth was then immediately added to a final volume of 1 ml, and the cells were incubated at 37 °C on a rotating rack for 1 hour. With both types of transformations, aliquots of 1-100 μ l of the culture were spread onto LB agar containing the appropriate antibiotics as well as IPTG (100 mM) and X-gal (40 μ g/ml) when blue-white selection was used to screen for the presence of insert DNA. The plates were then incubated overnight at 30 or 37 °C, where indicated.

2.2.4 Isolation of chromosomal DNA from *Streptomyces*

Chromosomal DNA isolation was carried out using Procedure 3 described by Hopwood *et al.* (1985). Solutions were scaled down appropriately for small-scale preparations.

2.2.5 Preparation of *Streptomyces* protoplasts

Protoplasts were prepared according to the procedure described by Hopwood *et al.* (1985), except that cultures were routinely grown in TSB or R2YE broth supplemented with 0.5% glycine.

2.2.6 Transformation of *Streptomyces*

Transformation of *Streptomyces* protoplasts was carried out according to the procedure described by Hopwood *et al.* (1985).

2.2.7 Conjugation into *Streptomyces* from *E. coli*

Conjugation was performed according to the procedure described by Kieser *et al.* (2000). Briefly, an overnight culture of *E. coli* ET12567/pUZ8002 harbouring the *oriT*-containing plasmid to be transferred was diluted 1:100 into fresh LB broth containing the appropriate antibiotics, and was incubated at 37 °C until the OD₆₀₀ = 0.4-0.8. The cells were washed twice with LB broth without antibiotics, and were resuspended in 0.1 volume of LB broth. When *Streptomyces* spores were used for conjugation, the spores (~10⁸) were suspended in 500 µl 2 × YT broth, heated-shocked for 10 minutes at 50 °C, and then mixed with 500 µl of the *E. coli* cells in a 15 ml round bottomed plastic tube (Corning). For conjugation into mycelial fragments, mycelia from a 3-5 day old R2YE plate were scraped from the cellophane disk into a glass homogenizer containing 1-2 ml of 20% glycerol, and were then homogenized. The mycelial fragments (500 µl) were then mixed with 500 µl *E. coli* cells. In both cases, the *E. coli*-*Streptomyces* mixture was centrifuged briefly in a clinical centrifuge at 3000 rpm, and after decanting the supernatant the pellets were resuspended in 200-300 µl LB broth. The mixture was then

plated onto MS agar plates containing 10 mM MgCl₂, and the plates were incubated at 30 °C for 16-24 hours. The agar was overlayed with 1 ml of solution containing nalidixic acid and the appropriate antibiotic for selection of the incoming plasmid, and the plates were further incubated for 3-4 days or until growth was apparent. Single exconjugants were then picked and re-streaked 1-2× onto MS agar containing nalidixic acid and the appropriate antibiotic.

2.3 DNA Purification and Analysis

2.3.1 Cloning and digestion of DNA

Plasmid and chromosomal DNA and PCR products (see below) were digested with restriction endonuclease enzymes as suggested by the manufacturer (Roche or New England Biolabs) and as described by Sambrook *et al.* (1985). Where indicated, non-homologous A-residues added onto 3' ends of PCR products by Taq polymerase were removed using T4 DNA polymerase. Reactions contained PCR product (~1 µg), 0.1 mM dNTPs, 5 mM MgCl₂, and 1 U of T4 DNA polymerase (Roche), and were incubated at 12 °C for 15 minutes before heating to 75 °C for 10 minutes in order to inactivate the enzyme. Blunting reactions where Klenow (Roche) enzyme was used were performed as described (Roche) by incubating DNA (~1 µg) with 1 mM dNTPs, 1× Filling buffer (50 mM Tris-HCl, pH = 7.5; 10 mM MgCl₂; 1 mM DTT; 50 µg/ml BSA) and 1 U of Klenow for 15 minutes at 37 °C, followed by 10 minutes at 65 °C. Ligation reactions were preformed in 5-10 µl volumes in 1 mM ATP and 1× ligation buffer (50 mM Tris-HCl, pH = 7.6; 10 mM MgCl₂; 5% PEG 8000; 1 mM DTT). The insert to vector ratio used was typically ~1-3:1 for sticky-end ligations, and ~4:1 for blunt-sticky-end ligations.

Reactions were initiated by the addition of 0.5 U of T4 DNA ligase (Roche), and were incubated at 15 °C for 4-24 hours in the case of sticky-end ligations, and at room temperature for 4 hours followed by 15 °C for 12-24 hours in the case of blunt-sticky-end ligations. Ligation of PCR products with 3' A overhangs into the pCR2.1 TOPO vector were set up and incubated as described by the TOPO TA cloning kit manufacturer (Invitrogen).

2.3.2 Polymerase chain reaction (PCR)

Polymerase chain reaction was employed to amplify DNA fragments to be used in cloning reactions and to be used as probes for northern and Southern analysis and for S1 mapping. It was also used as a method for screening plasmid clones for the presence of insert DNA. Reactions were performed in 50-100 µl volumes in 0.6 ml tubes (Rose) using the Techne PHC-2 thermocycler, or in 0.2 ml thin-walled tubes (Rose) using the Perkin Elmer GeneAmp PCR System 2400. Amplification was accomplished using either the EXPAND™ Long Template or High Fidelity enzymes, and typical reaction mixtures consisted of 1 mM MgCl₂, 0.2 mM dNTPs, 1× Reaction Buffer (Buffer 2 or 3 for Long Template enzyme, Buffer 3 for High Fidelity enzyme), 40 pmol of each oligonucleotide primer (see Table 2.5), 4% DMSO, 2.5 U of enzyme and 1-10 ng of plasmid DNA. For reactions in which chromosomal DNA was used as the template, 1 µg of DNA was first denatured by incubating at room temperature for 10 minutes in the presence of 0.4 N NaOH and 0.4 mM EDTA, followed by DNA precipitation and resuspension in H₂O. Sterile mineral oil (3 drops) was used to overlay reactions when the

Table 2.5: Oligonucleotide primers used in this study

Primer	Sequence (5' - 3')*	Region of Homology	Use
BKL54	CCGCGCTTCGCCACCGGT	16S rRNA DNA sequence	16S rRNA northern probe.
BKL62	CGGCGAATTCGTCTGCAACCAAGGAGCGC	Internal <i>bldG</i> sequence, nt +238 to +255**	PCR to generate probe for 3' S1 mapping of <i>bldG</i>
BKL63	GTGAGGCCGGTGATACG	Complementary to internal <i>bldG</i> sequence, nt +271 to +287	PCR amplification of <i>bldG</i> northern probe; sequencing.
BKL64	GCCCAAGCTCGGTGAGC	Internal <i>bldG</i> sequence, nt +81 to +97	PCR amplification of <i>bldG</i> northern probe; sequencing.
BKL65	CGCGGAATTCGCGGTCCAGGAGCATGC	Upstream of <i>bldG</i> , nt -241 to -225; includes <i>EcoRI</i> site	PCR; sequencing of <i>bldG</i> mutants.
BKL81	GCGGGAATTCGGTCCGAGCTCGTGAAACG	Internal <i>bldG</i> sequence, nt +102 to +118; includes <i>EcoRI</i> site	Sequencing of <i>bldG</i> mutants.
BKL83	GCGCAAGCTTGGGTGAATGCGGCGGTC	Complementary to sequence downstream of <i>orf3</i> , nt +455 to +471***	PCR amplification of <i>orf3</i> northern probe.
BKL92	CGCCACCGCTTCCTCGA	Complementary to internal <i>bldG</i> sequence, nt +314 to +330	Sequencing of <i>bldG</i> mutants.
BKL104	GCAAAACCTCATACAGAAAAATTC	Sequence flanking the polylinker region of pJ6902	Sequencing of inserts in pJ6902 and derivatives
BKL105	CACGCGGAACGTCCGGGCTTGAC	Sequence flanking the polylinker region of pJ6902	Sequencing of inserts in pJ6902 and derivatives
DBG3	GCGCTCTAGAGTTTCGACGGTGCGCATG	Complementary to sequence downstream of <i>bldG</i> , nt +451 to +467; includes <i>XbaI</i> site	PCR of insert for <i>bldG</i> complementation plasmid
DBG4	CGCGGATCCGACCTGTCCCTGTGCGAC	<i>bldG</i> sequence, nt +4 to +20; includes <i>BamHI</i> site	Sequencing of <i>bldG</i> mutants.
DBG5	GGTAAGCTTTTCAGTCGGTGGCGCCACCGCTT	Complementary to internal <i>bldG</i> sequence, nt +320 to +342; includes <i>HindIII</i> site	PCR amplification of <i>bldG</i> for cloning into pMAL-c2X
DBG8	GCGCAAGCTTGTCCGTACCGCCGTCT	internal <i>orf3</i> sequence, nt +43 to +59; includes <i>HindIII</i> site	PCR amplification of <i>orf3</i> northern probe.
DBG12	GCGCGAATTCGTGCGCGGTGGCGGACGAC	Upstream of <i>bldG</i> , nt -450 to -434; includes <i>EcoRI</i> site	PCR of insert for <i>bldG</i> complementation plasmid; PCR amplification of <i>bldG</i> 5' end for construction of <i>bldG</i> in-frame deletion vector.
DBG14	CGTCAATTTCCGCCACCGA	Complementary to internal <i>bldG</i> sequence, nt +53 to +70	PCR to generate S1 mapping probe for <i>bldG</i>
DBG15	GTCGACAGGGACAGGTC	Complementary to internal <i>bldG</i> sequence, nt +4 to +20	Primer extension analysis of <i>bldG</i>
DBG17	GCGCCTCCAGCTCGTCGA	Complementary to internal <i>orf3</i> sequence, nt -287 to +304	PCR to generate probe for 3' S1 mapping of <i>bldG</i>

Primer	Sequence (5' - 3')	Region of Homology	Use
DBG19	<u>GCGCAAGCTT</u> TCAGATCGGTGGCAGCAC	Complementary to internal <i>orf3</i> sequence, nt +415 to +432, includes <i>HindIII</i> site	PCR amplification of 3' end of <i>bldG</i> for construction of <i>bldG</i> in-frame deletion vector
DBG20	ATGGACCTGTCCCTGTCGAC	Internal <i>bldG</i> sequence, nt +2 to +20, changes the <i>bldG</i> start codon from GTG to ATG	PCR amplification of <i>bldG</i> for cloning into pMAL-c2X
DBG22	<u>GCGGGAATTC</u> TCCGAACTGCGGGCGTGCT	Complementary to sequence downstream of <i>bldG</i> , nt +424 to +443; includes <i>EcoRI</i> site	PCR amplification of <i>bldG</i> for cloning into pAlter-Ex2
DBG30	<u>GCGCTCTAGAT</u> CGGTGCGAGGAAGCGGTGG	Internal <i>bldG</i> sequence, nt +310 to +328, includes <i>XbaI</i> site	PCR amplification of <i>bldG</i> 3' end for construction of <i>bldG</i> in-frame deletion vector
DBG31	<u>GCGCTCTAGAA</u> CCGACCTCGACGACCGT	Complementary to internal <i>bldG</i> sequence, nt +53 to +70, includes <i>XbaI</i> site	PCR amplification of 5' end of <i>bldG</i> for construction of <i>bldG</i> in-frame deletion vector
DBG33	<u>GCGCTCTAGAT</u> CCCATGCCATTGATCGTGAA	Complementary to sequence downstream of <i>orf3</i> , nt +558 to +578; includes <i>XbaI</i> site	PCR amplification of <i>orf3</i> for cloning into <i>Streptomyces</i> overexpression vector
DBG34	<u>GCGCCATA</u> TGCCACCCTGCGAACTCCGT	Internal <i>orf3</i> sequence, nt +1 to +21; includes <i>NdeI</i> site	PCR amplification of <i>orf3</i> for cloning into <i>Streptomyces</i> overexpression vector
DBG35	<u>GCGCCATA</u> TGGACCTGTCCCTGTCGAC	Internal <i>bldG</i> sequence, nt +2 to +20; includes <i>NdeI</i> site which changes start codon from GTG to ATG	PCR amplification of <i>bldG</i> for cloning into <i>Streptomyces</i> overexpression vector
DBG40	GCCCTGTGGCTCGAGGAA	Complementary to internal <i>bldG</i> sequence, nt +160 to +178, contains a single non-homologous C residue (underlined)	Site-directed mutagenesis of <i>bldG</i>
DBG41	TCAGATCGGTGGCAGCACCCGCCGACGCGGTGG GCCAGGTgttagctggagctgcttc	Upper case: complementary to internal <i>orf3</i> sequence, nt +394 to +432, includes stop codon; Lower case: complementary to flanking sequence of <i>aac(3)/IV + oriT</i> cassette	PCR amplification of <i>orf3</i> disruption cassette
DBG42	ATGCCACCCTCGAACTCCGTTTCAGCGCGCT GCCCGAGAAattcegggagctcgacc	Upper case: internal <i>orf3</i> sequence, nt +1 to +39, includes start codon; Lower case: flanking sequence of <i>aac(3)/IV + oriT</i> cassette	PCR amplification of <i>orf3</i> disruption cassette
JST7	CGCCAGGGTTTCCACAGTCACGAC	Sequence flanking the polylinker region of pSET152	PCR amplification of inserts in pSET152 and derivatives
JST8	GAGCGGATAACAATTTCACACAGGA	Sequence flanking the polylinker region of pSET152	PCR amplification of inserts in pSET152 and derivatives
JWA1	TCAGTCCACCCACCAGC	Complementary to internal <i>bldG</i> sequence, nt +186 to +202	Sequencing of <i>bldG</i> mutants
JWA5	CACCGAGTCTGTCACTG	Upstream of <i>bldG</i> , nt -160 to -144	PCR, sequencing of <i>bldG</i> mutants

Primer	Sequence (5' - 3')	Region of Homology	Use
JWA6	GTTTCGACGGTGGCCATG	Complementary to sequence downstream of <i>bldG</i> , nt +451 to +467	PCR, sequencing of <i>bldG</i> mutants
JWA17	GGTACGGACGTGCTCGG	Complementary to internal <i>orf3</i> sequence, nt +35 to +51	PCR to generate probe for S1 mapping of <i>bldG-orf3</i> intergenic region; sequencing
JWA18	<u>GCGCAAGCTT</u> GGCCACCGACTGACGACC	Sequence in <i>bldG-orf3</i> intergenic region, nt +331 to +347**; includes <i>HindIII</i> site	PCR to generate probe for S1 mapping of <i>bldG-orf3</i> intergenic region
JWA20	<u>GCGCAAGCTT</u> GGGATCGATCGGGTCCG	Upstream of <i>bldG</i> , nt -178 to -194; includes <i>HindIII</i> site	PCR to generate probe for S1 mapping of <i>bldG</i>
KC11	<u>GCGCGGATCC</u> GTGGACCTGTCCCTGTCGAC	<i>bldG</i> sequence, nt -6 to +20	PCR amplification of mutant <i>bldG</i> sequence from pAU320
KC12	<u>GCGCTCTAGACTCGGAACTGCGGGCGTGCT</u>	Same as DBG22, except that it includes an <i>XbaI</i> site	PCR amplification of mutant <i>bldG</i> sequence from pAU320
WSC2	CATTGCCATACGGAGTTCCGGATGAGC	Chloramphenicol resistance gene sequence	Site-directed mutagenesis in pAlter-Ex2 to restore chloramphenicol resistance

*Non-homologous extensions are underlined, and engineered restriction sites are indicated in bold.

** Nucleotide positions are relative to the *bldG* start codon

*** Nucleotide positions are relative to the *orf3* start codon

Techne PHC-2 thermocycler was used, whereas a heated lid was used with the Perkin Elmer thermocycler to avoid evaporation of the reaction mixtures. A typical PCR program consisted of denaturing for 5 minutes at 95 °C, followed by 30 cycles of denaturing for 30 seconds at 95 °C, primer annealing for 30 seconds at 50-65 °C (~5 °C below the primer T_m , see 2.3.10), and extension for 1 minute at 68 °C.

PCR performed specifically for the PCR-targeted mutagenesis of *orf3* was carried out using a gel-purified 1383 bp *EcoRI/HindIII* disruption cassette from pIJ773 (See Table 2.3 and Section 2.3.4) as the template and the primers DBG41 and DBG42 (Table 2.5). Reactions consisted of 50 ng of template in a 50 µl reaction volume along with 50 µM of dNTPs, 50 pmol of each primer, 5% DMSO, 1× Reaction Buffer 2 (Roche) and EXPAND™ Hi-Fidelity polymerase (2.5U). Cycle conditions consisted of a 2 minute denaturation step at 95 °C, followed by 10 cycles of denaturation at 95 °C for 45 seconds, annealing at 50 °C for 45 seconds, and extension at 72 °C for 1 minute 30 seconds. This was followed by 15 cycles with the annealing temperature increased to 55 °C. The program was then concluded with an elongation step for 5 minutes at 72 °C, and the resulting PCR product was purified using a PCR purification kit according to the manufacturer's instruction (Qiagen), except that the DNA was eluted from the spin column using 12 µl of 10 mM Tris-HCl, pH = 8.5 instead of 50 µl.

2.3.3 Agarose gel electrophoresis of DNA

DNA fragments between 0.7 and 10 kb were separated by electrophoresis on 1% agarose, 1× TBE (90 mM Tris; 89 mM boric acid; 2.5 mM Na₂EDTA) gels. Lambda DNA (Roche) digested with *Pst*I was generally used as a molecular weight marker. DNA

fragments between 0.2 and 0.7 kb were electrophoresed on 2% agarose, 1× TBE gels, with Molecular Weight Marker V (0.25 µg, Roche) as the marker. Samples were prepared for electrophoresis by adding 1/5 volume of loading dye (0.25% bromophenol blue; 40% sucrose) which increased the density of the sample as well as provided a visual means of monitoring the separation of the DNA. After electrophoresis, DNA bands in the gel were visualized by incubating the gel at room temperature in 1× TBE containing ethidium bromide for at least 5 minutes, and then illuminating the gel under UV light.

2.3.4 DNA purification from agarose gels

DNA fragments larger than ~0.2 kb were purified from agarose gel by the Trough purification method (Zhen & Swank, 1993). The agarose gel (1-2%) was prepared using 1× TAE (40 mM Tris-acetate; 1 mM EDTA) and was poured to a thickness of 0.5-1 cm by taping around the edges of a glass plate. Loading dye was added to the samples, and electrophoresis was carried out at 107 V in 1× TAE buffer containing ethidium bromide. After visualizing the DNA under UV light, a trough (~0.5 cm²) was cut out of the gel immediately ahead of the desired DNA fragment, and the gel was placed back into the electrophoretic tank. The buffer level was adjusted such that it was half way up the edge of the gel, and the trough was filled with a solution containing 15% PEG 8000 and 0.5 µg/ml ethidium bromide in 1× TAE. A voltage of ~136 V was next applied to the gel, and migration of the DNA fragment into the solution in the trough was followed using a hand-held UV illuminator (Mineralight® Lamp UVSL-25; Ultra-Violet Products, Inc.). Once all of the DNA was present in the trough, electrophoresis was stopped, and the trough solution was transferred to a 1.5 ml microfuge tube. The solution was extracted

1× with phenol/chloroform and 1× with chloroform, and the DNA was precipitated by adding 1/10 volume sodium acetate and 2 volumes of 95% ethanol. The DNA pellet was then washed with 70% ethanol and resuspended in either TE buffer or H₂O.

DNA fragments that were used as templates for PCR for PCR-targeted mutagenesis were gel-purified using the Qiagen gel extraction kit as per the manufacture's instruction.

2.3.5 Polyacrylamide gel electrophoresis of DNA

DNA fragments between 0.2 and 0.7 kb were separated by electrophoresis at 200 V on 5% polyacrylamide (29:1 acrylamide:N,N'-methylene bisacrylamide, BioRad), 1× TBE gel. For fragments between 0.2 and 0.5 kb in size, Molecular Weight Marker V was used as the marker, whereas Molecular Weight Marker III (0.25 µg, Roche) was used for fragments larger than 0.5 kb. Loading dye was added to the samples as described above, and DNA bands in the gel were visualized by staining with ethidium bromide followed by illumination with UV light.

2.3.6 DNA purification from polyacrylamide gels

DNA fragments smaller than ~0.7 kb were purified from polyacrylamide gels using the "Crush and Soak" method described by Sambrook *et al.* (1989). The DNA of interest was electrophoresed in the gel as described above, and after staining with ethidium bromide, the band was excised from the gel and was placed into a 1.5 ml microfuge tube. Next, the gel slice was crushed into small pieces, elution buffer (0.5M ammonium acetate; 1 mM EDTA) was added (1-2 volumes), and the tube was incubated overnight at 37 °C on a rotating rack. The gel pieces were pelleted by spinning the tube

in a microfuge for 1 minute at 13 000 rpm, and the supernatant was transferred to a new 1.5 ml microfuge tube. The gel pieces were then washed with an additional 0.5-1 volumes of elution buffer, and DNA in the pooled supernatants was precipitated by the addition of 2 volumes of 95% ethanol and 2 μ l glycogen (40 μ g; Roche). The resulting DNA pellet was then washed with 70% ethanol and resuspended in 1-20 μ l H₂O.

2.3.7 DNA Sequencing

Manual sequencing of DNA was performed using the chain termination method (Sanger *et al.*, 1977) as modified for use with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB). Reactions were set up as described by the kit manufacturer, and typical reaction mixtures consisted of 50-500 pmol of template DNA; 20 mM Tris-HCl, pH = 9.5; 6 mM MgCl₂; 2 pmol of oligonucleotide primer, 2 μ M dNTPs, 0.02 μ M ³³P-ddNTPs and 8 U Thermo Sequenase™ enzyme. Reactions were performed in either 0.6 ml tubes when using the Techne PHC-2 Thermocycler, or in 0.2 ml thin-walled tubes when using the Perkin Elmer GeneAmp PCR System 2400 Thermocycler, and typical conditions consisted of a denaturing step of 5 minutes at 95 °C followed by 30 cycles of denaturing for 30 seconds at 95 °C, primer annealing for 30 seconds at 50-65 °C (~5 °C below the primer T_m) and extension for 1 minute at 72 °C. Loading dye (98% deionized formamide; 10 mM EDTA, pH = 8; 0.025% bromophenol blue; 0.025% xylene cyanol) was then added (4 μ l), and 3-5 μ l of each reaction was heated for 5 minutes at 95 °C and loaded onto a 6% polyacrylamide sequencing gel [19:1 acrylamide:N,N'-methylenebisacrylamide (BioRad); 8.3M urea. 1× TBE]. The samples were separated by electrophoresis at 35-40 watts for 1.75-3 hours, and the gel was

transferred to Fisherbrand thick chromatography paper and was dried for 2 hours at 80 °C on a gel dryer (Bio-Rad) connected to a Savant pump. The dried gel was then exposed overnight to a phosphorscreen, and the sequence was visualized by scanning the screen in a phosphorImager (Molecular Dynamics Model 445 SI), and analyzing the scanned image using Imagequant™ software.

2.3.8 Southern Analysis

Southern analysis was performed according to the procedure described by Hopwood *et al.* (1985), as a modification of the Southern procedure (Southern, 1975). DNA samples were electrophoresed along with the molecular weight marker (λ PstI DNA or Marker III) at 23-60 V on a 1% agarose, 1% TBE gel until the bromophenol blue indicator dye reached the end of the gel. After staining the gel with ethidium bromide to visualize the DNA as described in 2.3.3, the gel was incubated for 2 × 10 minutes at room temperature in 0.25 M HCl with gentle shaking, followed by incubation in denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 2 × 15 minutes at room temperature with shaking in order to denature the DNA in the gel. After rinsing the gel three times in distilled H₂O, the gel was neutralized by incubating for 20 minutes in neutralizing solution (3 M NaCl; 0.5 M Tris-HCl, pH=7.5).

The transfer apparatus was set up such that a piece of Fisherbrand thick chromatography paper was wrapped around a glass plate, with the ends of the paper submersed into a reservoir of 20× SSC buffer (3 M NaCl; 0.3 M tri-sodium citrate). A second, smaller piece of chromatography paper was placed on top of the first, and both were soaked with 20× SSC buffer. After removing excess buffer from the

chromatography papers, the gel was placed well-side down onto the papers, and the membrane (Hybond™-N, Amersham) cut to the same size of the gel was placed on top of the gel. Two pieces of chromatography paper that were slightly smaller than the gel were then placed on top of the membrane, followed by a stack of paper towel (~2.5 inches thick), a glass plate, and finally a solution bottle (~ 500 g) to weigh down the entire apparatus. The transfer apparatus was left to sit overnight, and after marking the position of the wells on the membrane, the DNA was cross-linked to the membrane at 150 mJoules using a Bio-Rad GS Gene Linker.

2.3.9 Labeling of DNA probes

DNA fragments that were used as probes for DNA hybridization (Southern analysis) and for detecting specific RNA transcripts (northern analysis) were internally labelled using the random primer labeling procedure described by Feinberg & Vogelstein (1983) and modified by Roche. The DNA sample (2-9 µl) was mixed with H₂O to a final volume of 9 µl in a 1.5 ml microfuge tube, and the mixture was heated to 95 °C for 5 minutes. After placing the tube on ice, 2 µl of hexanucleotide mix (Roche), 3 µl of 0.125 mM dNTPs (dATP, dGTP, dTTP) and 5 µl (50 µCi) of α -³²P-dCTP (Amersham) were added to the denatured DNA sample. The labeling reaction was initiated by the addition of 2 U of Klenow enzyme (Roche), and the tube was incubated at 37 °C for 1-2 hours. The unincorporated nucleotides were removed from the labeling reaction using a Micro Biospin® 6 chromatography column (Bio-Rad) as described by the manufacturer, and 1 µl of probe was Cerenkov counted in a Beckman LS 3801 scintillation counter.

DNA fragments that were used as probes for 5' S1 mapping (see Section 2.4.3), and oligonucleotides used as probes for northern analysis and as primers for primer extension analysis (see Sections 2.4.2, 2.4.4) were labeled at the 5' ends using the end-labeling procedure of Chaconas & van de Sande (1980) which was modified by Roche. Typically, reactions were carried out in a 10 μ l volume and consisted of 1-3 μ l of DNA (10-50 pmol of oligonucleotide primer) 1 $\times$ kinase buffer (50 mM Tris-HCl, pH = 8; 10 mM MgCl₂; 5 mM DTT; 0.1 mM spermidine) and 5 μ l (50 μ Ci) of γ -³²P-ATP (4500 Ci/mmol; ICN). T4 polynucleotide kinase (10 U/ μ l, Roche) was diluted 1/10 in kinase dilution buffer (50 mM Tris-HCl, pH = 7.6; 1 mM DTT; 0.1 mM EDTA; 50% glycerol), and 1 μ l of this was added to the reaction, which was incubated for 15 minutes at 37 °C. A second 1 μ l aliquot of diluted kinase enzyme was added, and after incubating the reaction for a further 15 minutes at 37 °C, the DNA probe was precipitated by adding 1/10 volume sodium acetate, 2 volumes 95% ethanol, and 2 μ l glycogen. For probes used in S1 mapping, the entire DNA pellet was Cerenkov counted as described above, and was redissolved in milli-Q H₂O to give 50 000 cpm/ μ l. For oligonucleotides used in primer extension analysis, the DNA pellet was redissolved in milli-Q H₂O to give 2.5 pmol/ μ l DNA, whereas for oligonucleotide probes used in northern analysis, the pellet was redissolved in milli-Q H₂O, and 1 μ l was Cerenkov counted as described above.

DNA fragments that were used as probes for S1 mapping of 3' ends of transcripts were labeled at their 3' ends using a procedure that was modified from the one described by Roche. DNA (5-10 μ l) was digested with an appropriate restriction enzyme, and then 1 μ l of 5 mM dNTPs (dATP, dGTP, dTTP), 2.5 μ l (25 μ Ci) of α -³²P-dCTP and 1 U of Klenow enzyme (Roche) were added, and reactions were incubated at room temperature

for 20 minutes. The DNA was then precipitated as described above and resuspended in milli-Q H₂O to give 50 000 cpm/ μ l.

2.3.10 DNA Hybridization

Southern and northern hybridizations were routinely carried out in glass hybridization bottles (Robbins Scientific) in 10 ml of hybridization buffer [3 $\times$ SSC; 4 $\times$ Denhardt's solution (0.08% polyvinylpyrrolidone, MW 360 000); 100 μ g/ml denatured salmon sperm DNA and 50% deionized formamide, when necessary]. Prehybridizations were carried out in a hybridization oven (Robbins Scientific) for at least 1-4 hours before the addition of labeled DNA probe (2 000 000 – 4 000 000 cpm/10 ml). The temperature of the oven was set at $\sim$ 25 $^{\circ}$ C below the melting temperature of the probe, which was calculated using the formula $T_m = 81.5^{\circ}\text{C} + 16.6\log M + 0.41(\% \text{ G+C}) - 500/n - 0.61(\% \text{ formamide})$, where M is the ionic strength (0.45 for 3 $\times$ SSC) and n is the shortest length of homology (Hopwood *et al.*, 1985). When oligonucleotide probes were used, the hybridization temperature was set at $\sim$ 5 $^{\circ}$ C below the probe melting temperature, which was calculated using the formula $T_m = 4(\text{G+C}) + 2(\text{A+T})$ (Hopwood *et al.*, 1985). Deionized formamide was included in the hybridization buffer when the calculated melting temperature was greater than 65 $^{\circ}$ C.

Labeled DNA probes were allowed to hybridize to the membranes for at least 8 hours, after which the membranes were washed 2 $\times$ 20-30 minutes with wash solution I (2 $\times$ SSC; 0.1% SDS) and 2 $\times$ 20-30 minutes with wash solution II (0.2 $\times$ SSC; 0.1% SDS). The membranes were wrapped in Saran Wrap, exposed to a phosphorscreen, and visualization of the image was as described in 2.3.7.

Hybridized probes were stripped from Southern membranes by placing the membrane in boiling 0.1% SDS, and allowing the solution to cool to room temperature. For stripping of northern blots, the membrane was incubated overnight in stripping solution (0.005 M Tris-HCl, pH = 8; 0.002 M Na₂EDTA; 0.1× Denhardt's reagent) at 65 °C in a shaking water bath. In both cases, stripping was repeated 1-2×, or until the radioactive signal was significantly reduced, and the stripped membranes were stored at -20 °C.

2.4 RNA Analysis

2.4.1 Isolation of RNA

RNA was isolated from *Streptomyces* using the procedure described by Hopwood *et al.* (1985), which is a modified version of the procedure described by Kirby *et al.* (1967). Briefly, *Streptomyces* cultures were grown on cellophane disks on the surface of R2YE plates, and at specific time points, the mycelia was scraped directly from the discs into universal bottles containing 5 ml 1× Kirby's mix (1% sodium triisopropylphenylmethane sulfonate; 6% 4-amino salicylate; 6% neutral phenol buffered in 50 mM Tris-HCl, pH = 8.3) and a depth of 1-2 cm glass beads (Fisher; 3mm). Plate cultures isolated at 15 and 18 hours post-inoculation were scraped into 1 universal vial each, whereas cultures isolated at 24, 36 and 48 hours post-inoculation were routinely scraped into 2 universal vials for each time point, and the resulting RNA for a given time point was later combined after DNase digestion (see below). The suspensions were vortexed for 4 × 30 second intervals, with the bottles being placed on ice in between each interval. Phenol/chloroform (5 ml) was then added, the mixtures were vortexed for 1 minute, and

were then transferred to 13 ml polypropylene tubes (Sarstedt). The phases were separated by centrifugation for 10 minutes at 8500 rpm in a Beckman J2-H5 centrifuge using the JA20 rotor, and the aqueous layer containing the nucleic acids was transferred to a new polypropylene tube. Extraction with phenol/chloroform was performed 2 additional times or until no interface remained, and the nucleic acids were precipitated by adding 1/10 volume of DEPC-treated sodium acetate and 1 volume of isopropanol. The samples were stored overnight at -70°C , and after thawing on ice, were centrifuged for 10 minutes at 8000 rpm as described above. The pellets were washed briefly with 95% ethanol, were resuspended in 450 μl DEPC-treated H_2O , and the dissolved samples were then transferred to RNase-free 1.5 ml microfuge tubes (Fisher). Removal of chromosomal DNA was accomplished by adding 1/10 volume of 10 $\times$ DNase buffer (0.5 M Tris-HCl, pH = 7.8; 0.05 M MgCl_2) and 2 separate aliquots of 7 μl of RNase-free DNase (Roche, 10 U/ μl) at 30 minute intervals. The tubes were incubated at room temperature for 1 hour in total, and were then extracted 2 $\times$ with equal volumes of phenol chloroform and 2 $\times$ with equal volumes of chloroform. The RNA was precipitated as described above, and the tubes were left on ice for 30 minutes before spinning at 14 000 $\times$ g for 10 minutes. After washing the pellets with 95% ethanol, DEPC-treated H_2O (100-200 μl) was added, and the samples were left to sit on ice overnight at 4°C to redissolve. Quantification of the RNA was by spectrophotometric analysis, and the integrity of the RNA was assessed by agarose gel electrophoresis. RNA samples were kept for long term periods by precipitating the RNA as described above and storing the tubes at -80°C .

2.4.2 Northern analysis

Northern analysis was performed according to the procedure described by Williams & Mason (1985). RNA samples (40 µg), as well as the molecular weight marker (Marker III, 1 µl), were denatured by dissolving in 2.5 µl (final volume) DEPC-treated milli-Q H₂O, followed by incubating for 1 hour at 50 °C in a solution containing 1 M glyoxyl, 50% DMSO and 10 mM Na₂HPO₄/NaH₂PO₄ (pH = 7). RNA loading dye (50% glycerol; 10 mM Na₂HPO₄/NaH₂PO₄ (pH = 7); 0.4% bromophenol blue; 0.4% xylene cyanol) was added to each sample, and the samples were electrophoresed at 60-72 V on a 1.25% agarose, 10 mM Na₂HPO₄/NaH₂PO₄ gel using a re-circulating buffer system. Once the bromophenol blue indicator dye had migrated 2/3 of the way down the gel, electrophoresis was stopped, and the RNA and marker were transferred to Hybond™-N (Amersham) membrane overnight using a standard capillary transfer. After marking the positions of the wells, the RNA was UV cross-linked to the membrane as described in 2.3.8, and the membrane was baked for 1 hour under vacuum at 80 °C in order to remove the glyoxyl. The marker lane was then cut off, and the marker and RNA-containing membranes were either used immediately for hybridization to DNA probes, or were wrapped in saran wrap and stored at -20 °C.

2.4.3 High resolution S1 nuclease mapping

S1 nuclease mapping was used for mapping of 5' and 3' ends of mRNA transcripts, and for examining the timing of expression of a particular gene over a given time period. The probes used were designed such that they overlapped the predicted transcription start or stop sites, and included non-homologous extensions at the 3' end

(for 5' S1 nuclease mapping) or at the 5' end (for 3' S1 nuclease mapping) in order to distinguish between full-length protection of the probe and probe-probe re-annealing. The presence of the non-homologous tail also ensured that divergent transcripts would not be detected, since the external label would be removed during the S1 nuclease digestion.

For both 5' and 3' S1 mapping, 40 μ g total RNA was dissolved in 3 μ l end-labeled probe (50 000 cpm/ μ l), and the mixture was dried down in a Speed Vac Concentrator (Savant). The probe and RNA were then redissolved in 20 μ l of S1 hybridization buffer (3.2 mM PIPES, pH = 6.4; 80% formamide; 0.4 M NaCl; 1 mM EDTA) and were heated to 80 °C for 20 minutes in a water bath. After vortexing the mixture to ensure that the RNA and probe were completely redissolved, the tubes were incubated for a further 10 minutes at 80 °C, and the temperature of the bath was then decreased slowly to a temperature ~5-10 °C above the calculated melting temperature of the probe as described in 2.3.10 (where $M = 0.4$ for S1 hybridization buffer). The reactions were left at this temperature for ~3 hours or overnight, in order to allow annealing of the DNA probe to the RNA transcript, and were then placed in ice. Digestion with S1 nuclease was performed by adding 400 U of S1 nuclease (Roche) in 300 μ l S1 digestion buffer [0.28 M NaCl; 30 mM CH₃COONa, pH = 4.4; 4.5 mM (CH₃COO)₂Zn; 20 μ g partially-cleaved denatured calf thymus DNA], and incubating the tubes at 37 °C for 45 minutes. After stopping the reactions by adding 75 μ l of S1 termination solution (2.5 M ammonium acetate; 0.05 M EDTA), the DNA/RNA duplex was precipitated for 30 minutes on ice, or overnight at -20 °C, by adding 1/10 volume sodium acetate, 2 volumes of 95% ethanol, and 2-3 μ l of glycogen (40-60 μ g; Roche).

The nucleic acids were pelleted, washed with 75% ethanol and then redissolved in 3-5 μ l of loading dye (see 2.3.5). Sequencing reactions were set up as described in 2.3.5 using one of the primers used to generate the probe in the case of 5' S1 mapping, or using a primer just upstream of the 3' end of the probe in the case of 3' S1 mapping. These reactions along with the samples were heated at 95 °C for 5 minutes, and then electrophoresed on a 6% polyacrylamide sequencing gel for 2-4 hours at 35-40 W constant power. The gel was then dried and exposed to a phosphorscreen for visualization.

2.4.4 Primer extension analysis

Primer extension analysis was performed according to the procedure described by Gabriella Keleman (personal communication) to map the position of transcription start sites. RNA (40 μ g) was mixed with end-labeled primer (5 pmol) in 1 $\times$ SB buffer [60 mM NH_4Cl ; 10 mM Tris-acetate, pH = 7.4; 6 mM 2-mercaptoethanol; (Hartz *et al.*, 1988)], heated to 90 °C for 5 minutes, and then transferred to a 75 °C water bath, which was slow-cooled to 55 °C. The reaction was left at this temperature for 1 hour, and the DNA/RNA duplex was then ethanol precipitated and resuspended in milli-Q H_2O . Extension of the primer was performed at 45 °C for 1 hour in a solution containing 1 $\times$ SB buffer, 15 mM Mg-acetate; 3 mM dNTPs; 17.5 U RNA Guard (Amersham) and 12.5 U AMV reverse transcriptase (Roche). Loading dye was added (see 2.3.5), and the reaction was evaporated at 80 °C for ~20 minutes. The entire reaction mixture was loaded onto a 6% sequencing gel along with sequencing reactions generated using the same

oligonucleotide used for the primer extension reaction, and the gel was run at 35-40 W for ~2 hours. Processing of the gel for visualization was as described above.

2.5 Site-Directed Mutagenesis

Site-directed mutagenesis was performed in order to change the serine residue at position 57 of BldG to an alanine residue. The template for mutagenesis was the *bldG* coding region cloned into the pAlter-Ex2 vector (pAU319, see Table 2.4), and mutagenesis was performed according to the Altered Sites *in vitro* Mutagenesis manual (Promega) using the oligonucleotide DBG40 to change the serine residue to an alanine residue, and the oligonucleotide WSC2 to restore chloramphenicol resistance to the vector (see Table 2.5). The resulting clone, designated pAU320, was then sequenced to confirm the presence of the desired S→A mutation in *bldG*.

2.6 Protein Overexpression and Purification

2.6.1 Overexpression of MBP-BldG and MBP-BldGS57A

BldG and BldGS57A (see above) were overexpressed in *E. coli* DH5α as N-terminal MBP-fusion proteins. In the case of MBP-BldG, small-scale pilot experiments were first performed as described by the pMAL-c2X instruction manual (Protocol A, NEB) to determine whether the fusion protein was being expressed and whether it was soluble. The only modification to the procedure was that release of proteins during sonication was not monitored using the Bradford assay. For large-scale expression of MBP-BldG and MBP-BldGS57A, a single colony of *E. coli* DH5α containing either pAU308 (for MBP-BldG overexpression; Table 2.4) or pAU321 (for MBP-BldGS57A

overexpression; Table 2.4) was used to inoculate 2 ml of L-broth containing 0.2% w/v glucose and 100 µg/ml ampicillin. The culture was incubated at 37 °C for 8-10 hours, then diluted 100 fold into 10 ml fresh L-broth + glucose and ampicillin, and incubated overnight at 37 °C. This overnight culture was next used to inoculate 1 L of L-broth + glucose and ampicillin, and incubation of the large-scale culture at 37 °C was until the OD₆₀₀ was 0.5-0.6. IPTG was then added at a final concentration of 0.3 mM to induce protein overexpression, and the culture was incubated at room temperature with shaking for 6 hours. The cells were then pelleted, and frozen overnight at -20 °C.

2.6.2 Purification of MBP-BldG and MBP-BldGS57A

Purification of MBP-BldG or MBP-BldGS57A was performed using amylose affinity chromatography as described by the resin manufacturer (New England BioLabs) with some modifications. Frozen cell pellets (see above) were thawed on ice, and resuspended in 50 ml of column buffer (20 mM Tris-HCl, pH=7.4; 200 mM NaCl, 1 mM EDTA) containing 1× Complete EDTA-free protease inhibitor cocktail (Roche). The cell suspension was sonicated on ice for 6 × 15 seconds using a 2.5 mm probe on low setting (Branson Sonifier 450), and the resulting cell lysate was centrifuged at 11 950 × g for 15 minutes at 4 °C. The supernatant containing soluble MBP-BldG was diluted 4 fold with column buffer and applied to 7 disposable columns (Bio-Rad) each containing 2 ml of amylose resin equilibrated in column buffer. The columns were each washed with 20 ml of column buffer before elution with column buffer containing 10 mM maltose. Fractions collected were analyzed by SDS-PAGE (see below), and in the case of MBP-BldG, fractions containing the fusion protein were pooled and dialyzed 3 times against 20

mM Tris-HCl, pH= 8.0, 25 mM NaCl at 4 °C before loading onto a MonoQ anion exchange FPLC column (Amersham) in order to further purify the protein as well as to remove any maltose that was still associated with the MBP tag. The protein was eluted from this column using a gradient from 25–500 mM NaCl in 20 mM Tris-HCl, pH = 8, and fractions containing the protein (as determined by SDS-PAGE; see below) were pooled and stored in small aliquots at –80 °C in the presence of 10% glycerol. In the case of MBP-BldGS57A, fractions eluted from the amylose columns containing the desired protein were pooled, and aliquots were stored at –80 °C in the presence of 10% glycerol.

2.6.3 Purification of BldG

Purified BldG protein was obtained by incubating purified MBP-BldG (~7.5 mg) with Factor Xa (50 µg) in amylose column buffer (see above) containing 0.05% SDS in order to remove the MBP tag. After incubating for 24 hours at room temperature, the reaction mixture was concentrated down to 500 µl using an Ultrafree-4 Centrifugal filter and tube (5K NMMW, Millipore), and was loaded onto a Superdex-75 FPLC column. Fractions eluted from the column were analyzed by SDS-PAGE, and those fractions containing untagged BldG protein as well as uncleaved MBP-BldG and MBP protein were combined and were incubated with amylose resin in column buffer overnight at 4 °C. The resin was then pelleted, and the supernatant was transferred to a clean tube, and an aliquot was analyzed by SDS-PAGE. The sample was then concentrated, and aliquots were stored at –80 °C in the presence of 15% glycerol.

2.6.4 Overexpression of His₁₀-ORF3

The ORF3 protein was overexpressed as an N-terminal 10× histidine tagged fusion protein in *E. coli* BL21(DE3)pLysS. A single colony of *E. coli* BL21(DE3)pLysS containing pAU318 (see Table 2.4) was used to inoculate 2 ml of LB broth containing 1% glucose, 30 µg/ml kanamycin and 25 µg/ml chloramphenicol. After incubation at 37 °C for 8-10 hours, the culture was diluted 100 fold in 10 or 20 ml of LB + glucose, kanamycin and chloramphenicol, and was incubated overnight at 37 °C. The overnight culture was then diluted 100 fold into 1 or 2 L of LB broth + glucose, kanamycin and chloramphenicol, and the cells were grown at 37 °C until the OD₆₀₀ was 0.5-0.6. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and the culture was further incubated at 37 °C for 2-3 hours. The cells were then pelleted and frozen overnight at -20 °C.

2.6.5 Purification of denatured His₁₀-ORF3

Purification of His₁₀-ORF3 from 1 L expression cultures (see above) was performed under denaturing conditions as described by the resin manufacturer (Novagen) with some modifications. Cell pellets stored at -20 °C were thawed on ice, resuspended in 20 ml lysing buffer (8 M urea; 0.1 M NaH₂PO₄; 0.01 Tris-HCl; pH = 8) and stirred for one hour at room temperature. The lysate was centrifuged at 11 952 × g for 20 minutes at 20 °C, and the supernatant containing soluble protein was added to 8 ml of a 50% Ni-NTA slurry in lysing buffer. The mixture was stirred for 1 hour at room temperature, and then loaded into 2 disposable columns (Bio-Rad). After washing the resin with ~10 column volumes of lysing buffer and ~10 column volumes of wash buffer (8 M urea; 0.1

M NaH₂PO₄; 0.01 M Tris-HCl; pH = 6.3), the bound fusion protein was eluted with elution buffer (8 M urea; 0.1 M NaH₂PO₄; 0.01 M Tris-HCl; pH = 4.5). Fractions collected were analyzed by SDS-PAGE, and those containing the desired protein were pooled and concentrated using an Ultrafree-4 Centrifugal filter and tube (5K NMMW, Millipore). Aliquots were then stored at -80 °C.

2.6.6 Purification of native His₁₀-ORF3

The His₁₀-ORF3 fusion protein was purified from 2 L expression cultures (see above) under native conditions by Ni-NTA affinity chromatography as described by the resin manufacturer, with some modifications. Frozen cell pellets were thawed on ice, and were lysed by resuspending in 10 ml of 1× Ni-NTA Bind Buffer (50 mM NaH₂PO₄, pH = 8; 300 mM NaCl; 10 mM imidazole) containing 1× Complete EDTA-free protease inhibitor cocktail (Roche). The lysate was centrifuged at 11 952 x g for 20 minutes at 4 °C, the supernatant was transferred to a clean tube, and was mixed with 8 ml of a 50% Ni-NTA resin slurry (prepared in 1× Ni-NTA Bind Buffer) for 1 hour at 4 °C. The mixture was then loaded into 2 disposable columns (Bio-Rad), and the resin was washed with ~8 column volumes of 1× Ni-NTA Wash Buffer (50 mM NaH₂PO₄, pH = 8; 300 mM NaCl; 20 mM imidazole). The resin-bound His₁₀-ORF3 was then eluted with 1× Ni-NTA Elution Buffer (50 mM NaH₂PO₄, pH = 8; 300 mM NaCl; 250 mM imidazole), and fractions collected were analyzed by SDS-PAGE. Those fractions containing the desired protein were pooled and concentrated (see above), and then dialyzed 3× at 4 °C against either 50 mM Na₂HPO₄/NaH₂PO₄, pH = 8; 300 mM NaCl or 20 mM Tris-HCl, pH = 7.4; 200 mM NaCl; 1 mM EDTA. Aliquots were then stored at -80 °C.

2.6.7 Preparation of *Streptomyces* cell-free extracts

Streptomyces cell extracts were prepared for western analysis and *in vitro* phosphorylation assays (see below) after growing for various lengths of time on the surface of cellophane disks on R2YE plates. Mycelia was scraped from the plates with a sterile spatula into a 1.5 ml microfuge tube, and lysing buffer [100 mM HEPES, pH = 7.2; 0.5 mg/ml lysozyme; 2× Complete EDTA-free protease inhibitor cocktail (Roche)] was added to 1 ml final volume. The suspension was incubated at 37 °C for 10 minutes and then sonicated on ice for 5 × 15 seconds on low setting using a 2.5 mm probe (Branson Sonifier 450). After centrifuging at 14 000 × g for 10 minutes at 4 °C, the supernatant containing soluble protein was transferred to a clean tube, and was aliquoted into small volume for storage at -80 °C. Protein concentration was determined as described below (Section 2.6.8).

2.6.8 Overexpression of BldG and ORF3 in *S. coelicolor*

BldG and ORF3 were overexpressed in *S. coelicolor* $\Delta bldG$ 1DB containing either the pAU316 or the pAU317 vectors, respectively (see Table 2.4), and were used in chemical cross-linking reactions (see Section 2.9.2). In each case, the genes were under control of the thiostrepton-inducible promoter *ptipA* (Murakami *et al.*, 1989). Briefly, ~200 μ l of mycelia for each strain were inoculated into 55 ml of R2YE broth containing 2.2% glycine and 0.05% antifoam 289 (Sigma) in spring flasks, and were incubated at 30 °C for ~15.5 hours. As a control, *S. coelicolor* $\Delta bldG$ 1DB containing the parent vector alone (pIJ6902) was cultured at the same time as the overexpression strains. Protein

expression was induced by the addition of 30 $\mu\text{g/ml}$ thiostrepton to each culture (including the control), and the flasks were further incubated at 30°C for 2 hours. The mycelia were pelleted and washed with 10.3% sucrose, and protoplasts were generated (see Section 2.2.4). The protoplasts were then lysed by resuspending in 0.5-1 ml of 1 $\times$ IP buffer (146 mM NaCl; 2 mM KCl; 10 mM HEPES; 5 mM EDTA; pH = 7.4) containing 1 $\times$ Complete EDTA-free protease inhibitor (Roche), and the lysates were transferred to 1.5 ml microfuge tubes and were treated with 2 $\times$ 7 μl aliquots of DNase (Roche, 10 U/ μl) for 1 hour on ice. The insoluble debris was pelleted, and the soluble fractions were transferred to new microfuge tubes. Protein extracts were then quantified and stored at 4 °C on ice.

Extract was also prepared from *S. coelicolor* M600 + pAU317 in which ORF3 was overexpressed for use in western analysis (see Section 2.7.3) and in affinity chromatography experiments (see Section 2.9.1). In this case, $\sim 10^9$ spores were inoculated into 20 ml of 2 $\times$ YT broth in a spring flask, and the flask was incubated at 30 °C for 7-8 hours to allow spore germination to occur. The germinated spores were pelleted by spinning at 1000 $\times$ g, resuspended in 3 ml of R2YE broth containing 6% PEG 8000, 2.2% glycine and 0.05% antifoam 289 (Sigma), and were sonicated briefly on low power in order to disperse the spores. The spores (1 ml) were then used to inoculate 55 ml of R2YE broth containing 6% PEG 8000, 2.2% glycine and 0.05% antifoam agent, and cultures were incubated at 30 °C with shaking for 14 hours. Induction of the culture with thiostrepton and preparation of crude extract was then carried out as described above.

2.6.9 Protein quantification

Quantification of purified proteins and *Streptomyces* total extracts was performed as described by Bradford (1976) using a Bio-Rad Protein Assay Kit as per the manufacturer's instruction. The standard curve relating absorbance at 595 nm to micrograms protein was used for quantification, and was constructed using Bovine Gamma Globulin (Bio-Rad).

2.7 Protein Analysis

2.7.1 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

Purified protein samples and crude cell extracts were analyzed by denaturing SDS-PAGE as described by Sambrook *et al.* (1985) with some modifications. Samples were prepared by adding 2× SDS loading dye (0.15 M Tris-HCl, pH = 6.8; 5% SDS; 24% glycerol; 12% 2-mercaptoethanol; 0.0024% bromophenol blue) to a final volume of 10-20 µl unless otherwise stated, and were denatured by heating at 90 °C for 5 minutes. SDS-PAGE broad range standards (1 µl, Bio-Rad) were used as a molecular weight marker, and were prepared in the same manner as the samples. The marker and samples were then electrophoresed on a 10-15% SDS-PAGE using either the Bio-Rad Mini Protean 3 or the Bio-Rad Protean 2 electrophoresis cells. The resolving gel consisted of 10-15% polyacrylamide (29:1 acrylamide: N,N'-methylene bisacrylamide), 0.375 M Tris-HCl, pH = 8.8 and 0.1% SDS, while the stacking gel consisted of 3.2% polyacrylamide (29:1 acrylamide:N,N'-methylene bisacrylamide), 0.125 M Tris-HCl, pH = 6.8 and 0.068% SDS. Tris-glycine-SDS buffer (0.05 M Tris; 0.38 M glycine; 0.1% SDS) was used as the

running buffer. Electrophoresis was carried out at room temperature or at 4 °C at constant voltage (200 V) for mini gels, or at constant current (30-40 mA) for large (16 × 20 cm) gels. Once the bromophenol blue indicator dye reached the bottom of the gel, the electrophoresis was stopped, and the gel was stained for 30 minutes to 2 hours at 37 °C in Coomassie Brilliant Blue protein stain [10% glacial acetic acid; 50% methanol; 0.001-0.002% w/v Coomassie Brilliant Blue (Bio-Rad)] followed by destaining at room temperature or 37 °C in a solution of 45% methanol and 10% acetic acid. Gels were then briefly soaked in 5% glycerol, placed onto Fisherbrand thick chromatography paper, and dried for 1 hour at 80 °C on a gel dryer (Bio-Rad) connected to a Savant pump.

2.7.2 Preparation of antibodies

Polyclonal antibodies against purified MBP-BldG and denatured purified His₁₀-ORF3 were generated by separating 1 mg of protein on a 15% or 12% SDS-PAG, respectively, for 5-6 hours at 30-40 mA, and then staining the gel for 20 minutes at 37 °C with 0.05% Coomassie Brilliant Blue in water. The bands corresponding to the desired proteins were excised, and the gel slices were cut into small pieces and frozen at -20 °C. After thawing, the gel slices were crushed, and 1× PBS buffer was added (to 0.75 ml total volume). The samples were then repeatedly passed through 18.5, 21 and 23 gauge syringe needles, and the resulting suspension was mixed with 0.75 ml of Freund's complete adjuvant for the first injection, and Freund's incomplete adjuvant for all other injections. Two rabbits were injected per protein at 4 week intervals for a total of 14 weeks, and cleared serum was prepared from blood samples taken 2 weeks after each

injection. The specificity and titre of the antibodies was then determined by western analysis (see below), and the antisera were aliquoted and stored at -80°C .

2.7.3 Western analysis

S. coelicolor crude extracts containing 40 μg total protein were mixed with SDS sample buffer (15-20 μl final volume), denatured by heating at 90°C for 5-10 minutes, and then separated on a large (16 $\times$ 20 cm) 12 or 15% SDS-PAG at 30-40 mA for 5-6 hours at 15°C . Kaleidoscope pre-stained standards (Bio-Rad) were used as the molecular weight markers as well as for monitoring both the progression of the proteins in the gel during electrophoresis and the efficiency of protein transfer to membrane. The gel was soaked in transfer buffer (19.2 mM Tris-HCl, pH = 8; 0.192 M glycine; 0.015% SDS; 20% methanol) for 30 minutes at room temperature on an orbital skaker, and transfer of the proteins to Sequi-Blot PVDF membrane (Bio-Rad) was set up overnight at 4°C and 20 V using a BioRad Transblot apparatus. After transfer, the membrane was washed 3 $\times$ 5 minutes with milli-Q H_2O and 1 $\times$ 5 minutes with TBS-T buffer (20 mM Tris-HCl, pH = 7.6; 140 mM NaCl; 0.5 % Tween-20), and then blocked for 1 hour at room temperature in 100 ml blocking buffer (10% skim milk in TBS-T buffer). To probe for BldG or ORF3, 50 ml of blocking buffer was removed and kept for use with the secondary antibody (see below), and anti-MBP-BldG at a dilution of 1 in 20 000, or anti-His₁₀-ORF3 at a dilution of 1 in 5 000, were incubated with the membrane for 1 hour at room temperature on an orbital shaker. The blot was washed 2 $\times$ 5 minutes and 1 $\times$ 15 minutes in TSB-T, and then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham) in blocking buffer at a dilution of 1 in 10 000. The secondary

antibody was detected by incubating the blot in Western Lightning Plus chemiluminescence reagent (Perkin Elmer) for 1 minute, and then exposing the blots to film for 1-60 minutes.

2.8 Phosphorylation Studies

2.8.1 *In vitro* phosphorylation reactions

In vitro phosphorylation assays were performed as a variation of the procedure described by Hong *et al.* (1991) in order to determine whether BldG could undergo phosphorylation. Purified MBP-BldG (1.5 μ g) was incubated with aliquots of *S. coelicolor* crude extract (15 μ g total protein isolated at various times post-inoculation) in phosphorylation buffer (10 mM Tris-HCl, pH = 7.4; 4 mM MgCl₂·6H₂O; 2 mM MnCl₂; 1 mM EDTA; 3 mM 2-mercaptoethanol). Reactions were also set up using purified MBP-BldGS57A (1.5 μ g) to determine whether the point mutation affected protein phosphorylation. Reactions were initiated by the addition of 5 μ Ci of γ -³²P-ATP or α -³²P-ATP, and were incubated at room temperature for 20 minutes before terminating with SDS sample buffer. The proteins were separated by SDS-PAGE at 200 V for ~45 minutes on a mini 10% SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue protein stain as described in 2.6.1 to view the proteins. To detect phosphorylation of BldG, the gel was dried and exposed to a phosphorscreen, and the image was visualized as described in 2.3.7.

To determine which nucleotide served as a phosphoryl group donor for BldG, nucleotide competition assays were performed by incubating 0.15 μ g MBP-BldG with 10

μ g crude extract and 1 mmol of cold ATP, CTP, GTP or UTP (Promega) for 1 hour prior to the addition of γ - ^{32} P-ATP. Reactions were then processed as described above.

2.8.2 Isoelectric focusing (IEF)

Isoelectric focusing was performed in order to detect phosphorylated BldG protein in *S. coelicolor* crude extracts. Extracts isolated at various times post-inoculation (40 μ g total protein) were suspended in 50% glycerol and were separated on a Ready Gel™ precast IEF gel, pH = 5-8 (Bio-Rad) using the Bio-Rad Mini Protean 3 electrophoresis system. IEF standards (Bio-Rad) were run along with the samples in order to follow the progression of the isoelectric focusing. The buffers used for separation were commercially prepared 10 $\times$ cathode buffer (Bio-Rad, 200 mM lysine; 200 mM arginine), and 10 $\times$ anode buffer (Bio-Rad, 70 mM phosphoric acid) that had been diluted to 1 $\times$ concentration prior to use. The gels were run at 4°C for 1 hour at 100 V, followed by 1 hour at 250 V, and then 30 minutes at 500 V. Proteins were then transferred to PVDF membrane for 1 hour at 4°C and 100 V using the Bio-Rad Mini Transblot apparatus and 0.7% acetic acid as the transfer buffer. Visualization of the BldG protein bands was by western analysis as described above, except that a 1 in 10 000 dilution of the anti-MBP-BldG antibodies was used. Densitometric analysis of the protein bands was performed using the computer software NIH Image version 1.63 available for downloading at the internet site <http://rsb.info.nih.gov/nih-image/>. Isoelectric points for native BldG and P-BldG were determined by calculating the net charge of the proteins at a given pH as described by Mosher *et al.* (1993). For simplicity, it was assumed that each ionizable group of the protein had the same pKa everywhere on

the molecule. The pKa values used for the calculations were as follows: C-terminal carboxy group = 3, aspartate = 3.2, glutamate = 4.1, cysteine = 9, tyrosine = 10, N-terminal α -amino group = 8, histidine = 6, lysine = 10, arginine = 12 (Henriksson *et al.*, 1995). The pKa₁ and pKa₂ values for phosphoserine that were used were 1 and 6, respectively (Towbin *et al.*, 2001).

2.9 Protein-Protein Interactions

2.9.1 Affinity chromatography

Affinity chromatography was used to determine whether BldG and ORF3 are able to interact and form a complex, and the procedure used was slightly modified from that described by Nussbaum-Shochat & Amster-Choder (1999). MBP-BldG (3 μ g) in 200 μ l amylose column buffer (see 2.6.2) containing 1 $\times$ Complete EDTA-free protease inhibitor was mixed with 50 μ l of a 50% amylose slurry (in column buffer) for 2-3 hours at 4 °C. The resin was washed with 3 $\times$ 1 ml column buffer, resuspended in 200 μ l column buffer + protease inhibitor, and mixed with either purified native His₁₀-ORF3 (3 μ g), with *S. coelicolor* J1501 cell extracts (400-500 μ g), or with *S. coelicolor* M600 + pAU317 cell extract (400 μ g) containing overexpressed ORF3 protein. The mixture was incubated at 4 °C with gentle agitation for 14-16 hours, after which the resin was washed 3 $\times$ with column buffer. Proteins bound to the resin were eluted by either incubating the resin in column buffer containing 10 mM maltose for 10 minutes on ice, and then pelleting the resin and transferring the supernatant to a clean tube, or by adding SDS-PAGE sample buffer to the resin and boiling the sample for at least 5 minutes. The presence of BldG and ORF3 in the supernatant was then analyzed by western analysis.

Binding was also assessed by first mixing MBP-BldG with *S. coelicolor* crude extracts for 14-16 hours at 4 °C in 200 µl of column buffer, and then adding 50 µl of a 50% amylose slurry and further incubating for 2 hours. Washing of the resin and elution of proteins was then performed as described above.

2.9.2 Chemical cross-linking

Chemical cross-linking experiments were performed to examine whether BldG and ORF3 can form a complex. The procedure used was a modified version of the procedure described by Alper *et al.* (1994), and involved the use of *S. coelicolor* $\Delta bldG$ 1DB extracts containing overexpressed BldG or ORF3 proteins (see 2.6.7). Reactions were carried out in 50 µl volumes containing 50 mM HEPES buffer, pH =7.5; 10 mM MgCl₂; 10% glycerol; 0.5 mg/ml BSA; 150 mM NaCl; and 1 mM DTT, and were performed in the presence and absence of 500 µM of ATP, ADP, CTP, GTP or UTP. Extract containing overexpressed BldG protein (20 µg total protein) was pre-incubated with overexpressed ORF3 extract (20 µg total protein) or with extract containing wild type levels of ORF3 (*S. coelicolor* $\Delta bldG$ 1DB + pIJ6902; 20 µg total protein) for 15 minutes at 30 °C in reaction buffer with or without nucleotides. Chemical cross-linker (BS3, Sigma) prepared fresh in 20 mM HEPES buffer (pH =7.2) was then added at a final concentration of 1 mM, and the reactions were further incubated at 30 °C for 30 minutes. Termination of the reactions was by the addition of 0.5 µl of 0.5 M lysine followed by 10 µl of SDS-PAGE loading dye. Samples were then concentrated, and complex formation involving BldG and ORF3 proteins was examined by western analysis.

2.10 DNA Microarray Analysis

2.10.1 RNA isolation

Global changes in gene expression were monitored by DNA microarray analysis in response to overexpression of BldG or ORF3 in wild type *S. coelicolor* M600. Spores of M600 + pAU316 (BldG overexpressing strain), M600 + pAU317 (ORF3 overexpressing strain) and M600 + pIJ6902 (control strain) were pre-germinated by inoculating 35 μ l of spore stock ($\sim 10^8$ spores) into 20 ml of 2 $\times$ YT broth in universal spring vials, and then incubating at 30 $^{\circ}$ C for 8 hours. After centrifuging at 1000 rpm for 10 minutes in an International Centrifuge with a swinging bucket rotor, the germinated spores were resuspended in 5 ml of NMMP medium, and were sonicated briefly using a 2.5 mm probe on low setting (Branson Sonifier 450) in order to disperse the spores. The suspensions were then divided evenly between six sialanized 250 ml spring flasks, five of which contained 50 ml of NMMP medium, and one of which contained 100 ml of NMMP medium (Time 0 sample, inoculum used was 2 $\times$ the amount used for 50 ml NMMP). The flasks (18 in total) were incubated at 30 $^{\circ}$ C for about 15.5 hours ($OD_{450} \sim 0.5$), after which time the respective cultures were combined together and redistributed into five new spring flasks, keeping 100 ml of each culture aside for the Time 0 (uninduced) sample. Induction of BldG or ORF3 production was by the addition of thiostrepton (30 μ g/ml final concentration), and cultures were incubated at 30 $^{\circ}$ C following induction for 15, 30, 45, 60 and 120 minutes. At each time point, a single flask for each culture was removed, and RNA isolation was performed by pelleting the mycelia at room temperature in a universal vial and then adding 5 ml of 1 $\times$ Kirby's Mix + 1-2cm

of glass beads, and proceeding as described in Section 2.4.1. Quantification of RNA and analysis of RNA integrity was performed as described previously (Section 2.4.1).

DNA microarray experiments were also performed using RNA isolated from the *bldGC103* mutant strain in which BldG was overexpressed. Mycelial stocks (100 μ l) of C103 + pAU317 and C103 + pIJ6902 (control strain) were inoculated into universal spring vials containing 5 ml of R2YE broth + 6% PEG 8000 (to enhance cell dispersal) + 0.05% antifoam 289, and were incubated at 30 °C for 24 hours. The 5 ml cultures were then subcultured into 25 ml of fresh R2YE broth + 6% PEG 8000 + 0.05% antifoam 289, and were incubated at 30 °C for another 24 hours, after which time aliquots (1 ml each) were subcultured into five spring flasks containing 50 ml R2YE + 6% PEG + 0.05% antifoam 289, and into one spring flask containing 100 ml of medium (inoculated with 2 ml of 24 hour culture). The flasks (12 in total) were incubated at 30 °C for 12 hours ($OD_{450} \sim 0.5$), at which time the cultures were combined and redistributed into new spring flasks (keeping 100 ml aside for Time 0 sample), and were induced with 30 μ g/ml (final concentration) of thiostrepton. Incubation of flasks and processing of cultures for RNA isolation was then carried out as described above.

To compare gene expression in wild type *S. coelicolor* and in a *bldG* mutant strain by DNA microarray analysis, strains were grown on the surface of cellophane disks on R2YE plates at 30 °C for 15, 18, 24, 36 and 48 hours, and RNA was extracted from the surface cultures as described previously (Section 2.4.1). *S. coelicolor* J1501 (wild type) spores (80 μ l/plate) and *bldGC103* mycelia (80 μ l/plate) were used to inoculate the plates, with each type of inoculum giving similar amounts of growth.

2.10.2 Microarray analysis

RNA isolated in the experiments described above (Section 3.1.1) was used as a template for the synthesis of fluorescently-labeled cDNA which was subsequently used in microarray hybridization experiments. The Time 0 (uninduced) samples from the first two experiments, and the 15 hour samples from the third experiment were used as the references to which subsequent samples were compared, and cDNAs generated from these samples were labeled with Cy-5 (green), while cDNAs generated from all other samples were labeled with Cy-3 (red). Total RNA (15 μ g) was first denatured along with 10 μ g of 72% GC content hexamers (total volume = 14 μ l) by incubating at 75 °C for 10 minutes and then snap-cooling on ice. Cy-3 or Cy-5-dCTP (2 μ l, Amersham Pharmacia) was then added to each reaction along with 14 μ l of a cocktail containing 6 μ l of 5 $\times$ Superscript II buffer, 3 μ l of DTT (0.1 M), 3 μ l of dNTPs (4 mM dATP, 4 mM dTTP, 10 mM dGTC, 0.2 mM dCTP), and 2 μ l of Superscript II enzyme (200 U/ μ l, Invitrogen). The reactions were incubated at room temperature for 10 minutes and then transferred to 42 °C for 2-3 hours. In initial experiments, the RNA was degraded following the 42 °C incubation by adding 1 μ l of 1 N NaOH, incubating for 10 minutes at 65 °C, and then neutralizing the reaction by adding 1 μ l of 1 N HCl, however this step was later omitted as it was deemed unnecessary. The labeled cDNA was purified by adding the reverse transcription reaction to a Microcon-10 filter (Amicon) containing 500 μ l of TE buffer, and then centrifuging for 20 minutes at 13 000 rpm. This step was repeated, and centrifugation was carried out until about 12 μ l of liquid remained, at which point the filters were inverted into fresh tubes, and the samples were recovered by centrifuging for 1-2 minutes.

Hybridization reactions were set up by mixing 6 μ l of the Cy-3 reaction with 12 μ l of the Cy-5 reaction, and then adding 1 μ l of polyA DNA, 4.5 μ l of 20 $\times$ SSC and 0.45 μ l of 10% SDS. The mixture was boiled for 2 minutes and then applied to a microarray under a cover slip, and the microarray was hybridized in a custom hybridization chamber submerged in a 65 $^{\circ}$ C water bath for 10-12 hours. The array was then washed in 1 $\times$ SSC, 0.3% SDS for ~30 seconds, followed by ~1 minute in 0.2 $\times$ SSC and ~1 minute in 0.05 $\times$ SSC, and was dried by centrifuging at 5000 rpm in a Beckman Allegra centrifuge. Scanning of the array was performed using a GenePix scanner (Axon Instruments) with GenePixPro 3.0 software, and data collected were analyzed using software available at <http://genome-www5.stanford.edu/MicroArray/SMD>.

2.11 Computer-Assisted Sequence and Structure Analysis

General raw sequence handling was done using the GeneTool 1.0 program (BioTools). Similarities between deduced protein products and known proteins in the database were detected using PSI-BLAST (three iterations) at the internet site <http://www.ncbi.nlm.nih.gov/BLAST>. Protein sequence alignments were generated using the ClustalW program (http://npsa-pbil.tbcg.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and were displayed using the Anthprot Program 5.0 (<http://antheprot-pbil.ibcp.fr/>). Protein structures were predicted using the SWISS-MODEL database (<http://epasy.org/swissmod/SWISS-MODEL.html>) and were viewed using the PBD Viewer program (<http://us.epasy.org/spdbv>). Potential RNA secondary structures, together with Δ G values, were determined using the Mfold 2.3 program with the folding temperature set at 30 $^{\circ}$ C, the standard growth temperature used

for these studies. The Mfold program is available at the internet site <http://www.ibc.wustl.edu/~zucker/rna/>. Analysis of the ORF3 protein sequence for conserved bacterial histidine kinase domains was done using the Prosite ProfileScan server located at the internet site http://www.isrec.isb-sib.ch/software/PFSCAN_form.html.

2.12 Scanning Electron Microscopy (SEM)

Analysis of the $\Delta bldG$ 1DB mutant strain containing pAU69 (see Table 2.4) was performed by growing the strain on R2YE agar for one week at 30 °C, and then mounting colonies on the surface of an aluminum stub using double-sided tape. The samples were viewed using the Philips FEI LaB6 environmental scanning electron microscope of the Biological Sciences Microscopy Unit at 3500 $\times$ magnification under a vacuum of 0.9 Torr.

Chapter 3:

Results

3. Results

The *bldG* locus was first identified by Dr. Wendy Champness (Champness, 1988) while isolating mutants that were blocked in the early stages of morphological and physiological development. Cloning of *bldG* was performed by Dr. Brenda Leskiw (Bignell *et al.*, 2000), and involved screening a phage library consisting of *S. coelicolor* M145 DNA fragments for the ability to restore pigmented antibiotic production and aerial hyphae formation to the *bldGC103* mutant. The DNA from one such lysogen was eventually cloned as a 2.5 kb *HindIII* fragment and sequenced by Jason Warawa, and a complete open reading frame as well as an upstream and a downstream partial open reading frame were identified. The complete open reading frame was found to encode a 113 amino acid protein with a predicted molecular weight of 12 321 Da, and since this open reading frame alone was able to restore aerial hyphae formation and pigmented antibiotic production to the C103 mutant, it was designated *bldG*. BLAST analysis indicated that BldG shows similarity to anti-anti-sigma factor proteins including RsbV and SpoIIAA of *B. subtilis*. Since genes encoding anti-anti-sigma factors are typically located next to genes encoding a cognate anti-sigma factor and a sigma factor, the remainder of the downstream open reading frame (designated *orf3*) sequence, as well as ~1 kb of sequence downstream of *orf3* was determined. The product of the *orf3* gene was found to be a 143 amino acid protein which showed similarity to anti-sigma factor proteins such as SpoIIAB and RsbW of *B. subtilis*. Unexpectedly, however, a sigma factor gene was not found downstream of *orf3*, or upstream of *bldG* (the genome sequencing project has revealed that a sigma factor gene is not present anywhere within the *bldG*-containing 40.54 kb region of the *S. coelicolor* chromosome that is present in

cosmid SCH5). Instead, partial open reading frames encoding a putative RNA helicase (Stoeck, 2001) and a putative pyrophosphate synthase were identified in the immediate vicinity of *bldG* and *orf3*.

The goal of this research was to investigate the role of the *bldG* locus in the regulation of morphological and physiological differentiation in *S. coelicolor*. Specifically, we were interested in determining how BldG functions to regulate these differentiation processes, and whether the ORF3 protein is involved along with BldG in controlling development in these organisms.

3.1 Sequence analysis of the *bldG* locus

3.1.1 Alignment of protein sequences

PSI-BLAST searches performed using the BldG protein sequence (with three iterations) revealed that BldG is similar to known or putative anti-anti-sigma factors from a number of organisms including *Bacillus* spp., *Staphylococcus* spp. and *Listeria* spp., as well as from other high G+C rich organisms like *Mycobacteria* spp. and *Thermobifida fusca*. The searches also pulled up eight other putative anti-anti-sigma factors from *S. coelicolor* itself, the most closely related of which, designated SC5F1.27c, showed 46% identity and 66% similarity to BldG. BldG was found to be 40% identical and 60% similar to RsbV, and 26% identical and 56% similar to SpoIIAA, of *B. subtilis*. The most closely related protein, however, was a putative anti-anti-sigma factor from *Streptomyces avermitilis* (SAV4614), whose amino acid sequence differed from that of BldG by only one residue (Figure 3.1.1). A hypothetical protein from the actinomycete *Thermobifida fusca* was also found to be closely related to BldG, showing 72% identity and 82%

Figure 3.1.1 Alignment of the *S. coelicolor* BldG protein with other anti-anti-sigma factors. The alignment was generated using the ClustalW Program (http://npsa-pbil.tbcg.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and was displayed using the AntheProt Program Version 5 (<http://antheprot-pbil.ibcp.fr/>). Red shading indicates identical residues in all of the sequences, while blue shading indicates identical residues in $\geq 75\%$ of the sequences, and green shading indicates identical residues in $\geq 50\%$ of the sequences. The consensus sequence for the alignment is given below the protein sequences, with identical residues in all sequences indicated by (*). The serine residue that is phosphorylated in SpoIIAA is indicated by (▼), while residues which have been shown to be important for SpoIIAA function are indicated by the black arrows. (◆) denotes the single residue that distinguishes BldG from the *S. avermitilis* putative anti-anti-sigma factor (SAV4614), and residues which are conserved in STAS domains are underlined, with the type of residue typically found at a particular position indicated as either h (hydrophobic, YFWLIVMA), l (aliphatic, LIVMA), s (small residue, SAGTVPNHD), u (tiny residue, GAS) or p (polar residue, STQNEDRKH) (Aravind & Koonin, 2002). A comparison of secondary structures in SpoIIAA (Kovacs *et al.*, 1998) and those predicted in BldG using the SWISS-Model database (<http://www.epasy.orf/swissmod/SWISS-MODEL.html>) is indicated above the alignment, with arrows representing β -strands, and cylinders representing α -helices. The SpoIIAA secondary structures are shown in white, while the BldG secondary structures are shown in yellow.

similarity to the BldG protein. Alignment of BldG with other anti-anti-sigma factor proteins (Fig. 3.1.1) indicated a number of regions with high conservation, including that encompassing the phosphorylatable serine residue of SpoIIAA and RsbV (Najafi *et al.*, 1995; Yang *et al.*, 1996). A number of point mutations in SpoIIAA that have been characterized and have been shown to affect SpoIIAA function are located within these highly conserved regions (Challoner-Courtney & Yudkin, 1993; Lee *et al.*, 2001), suggesting that these regions are important for the function of anti-anti-sigma factors in general. Among the conserved residues were amino acids that are also highly conserved in the cytoplasmic portion of anion transporters of the sulfate transporter family, and therefore define a shared domain between these two groups of proteins referred to as the STAS domain (for Sulfate Transporters and Anti-Sigma factor antagonists) (Aravind & Koonin, 2000). The significance of this shared domain, however, is currently unknown.

PSI-BLAST searches performed using the ORF3 protein sequence (three iterations) indicated that ORF3 is related to RsbW and SpoIIAB homologs from *Bacillus* spp. and *Staphylococcus* spp, as well as to hypothetical proteins found in organisms including *Listeria* spp., *Clostridium* spp. and *Chloroflexus* spp. ORF3 showed 28% identity and 43% similarity to the *B. subtilis* RsbW protein, and 26% identity and 42% similarity to the *B. subtilis* SpoIIAB protein. A hypothetical protein from *Streptomyces avermitilis* (SAV4615) was also found to show 76% identity and 86% similarity to the ORF3 protein. Interestingly, this hypothetical protein is encoded downstream of the *S. avermitilis* *bldG* ortholog (see Genome Project of *Streptomyces avermitilis* at the internet site <http://avermitilis.ls.kitasato-u.ac.jp/>) and probably represents the *orf3* ortholog in this organism. Sequence alignment of ORF3 with other anti-sigma factors (Fig. 3.1.2)

Figure 3.1.2 Protein sequence alignment of *S. coelicolor* ORF3 with anti-sigma factor homologs in other organisms. The alignment was generated using the ClustalW Program, and was displayed using the Antheplot Program (Version 5). Red shading indicates identical residues in all of the sequences, while blue shading indicates identical residues in $\geq 75\%$ of the sequences, and green shading indicates identical residues in $\geq 50\%$ of the sequences. The consensus sequence for the alignment is given below the protein sequences, with identical residues in all sequences indicated by (*). The SpoIIAB residue that has been shown to be important for SpoIIAB binding to SpoIIAA and σ^F is indicated by (◆) while signature residues of the N, D and G box regions conserved in bacterial histidine kinases are indicated by (▼).

[illegible]

B. licheniformis sigB regulator
B. subtilis RsbW
S. epidermidis RsbW
B. licheniformis SpoIIAB
B. subtilis SpoIIAB
S. avermitilis SAV4615
S. coelicolor ORF3
Consensus:

16AD	FT	FE	SV	RG	VE	UR	IE	1	AE	CS	F	T	1	UB	R	Q	R	C	1	FT	1	CH	TV	D	C	1	SE	1	IG	116
16BD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16CD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16ED	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16FD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16GD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16HD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16ID	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16JD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16KD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16LD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16MD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16ND	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16OD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16PD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16QD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16RD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16SD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16TD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16UD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16VD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R	Q	Q	C	1	PY	1	SH	TV	D	C	1	SE	1	IG	116
16WD	KN	EV	SI	RG	VS	UR	IE	1	AE	CS	F	T	1	FD	R															

B. licheniformis sigB regulator
B. subtilis RsbW
S. epidermidis RsbW
B. licheniformis SpoIIAB
B. subtilis SpoIIAB
S. avermitilis SAV4615
S. coelicolor ORF3
Consensus:

B. licheniformis sigB regulator
B. subtilis RsbW
S. epidermidis RsbW
B. licheniformis SpoIIAB
B. subtilis SpoIIAB
S. avermitilis SAV4615
S. coelicolor ORF3
Consensus:

[illegible]

indicated that as with the anti-anti-sigma factor alignment, there are specific amino acids which are highly conserved among most of the proteins, including ORF3. One of these residues, R20 of SpoIIAB, is thought to be important for SpoIIAB binding to both SpoIIAA and σ^F (Garsin *et al.*, 1998), and therefore may be important for protein-protein interactions in anti-sigma factors in general. Among the highly conserved residues are those that are also conserved in bacterial histidine kinases of two-component signal transduction systems. These residues represent key residues of the N, D and G box regions of histidine kinases which are thought to be involved in protein-protein interactions, Mg^{2+} binding and ATP binding, respectively (Garsin *et al.*, 1998; Stock *et al.*, 1995). Previous studies have demonstrated that both SpoIIAB and RsbW of *B. subtilis* possess serine kinase activity towards their cognate anti-anti-sigma factor (Dufour & Haldenwang, 1994; Min *et al.*, 1993), and that this interaction is important for the regulation of anti-anti-sigma factor and anti-sigma factor interactions. Alignment of the ORF3 protein sequence with that of RsbW and SpoIIAB, however, indicated that ORF3 contains only two of the five conserved amino acid residues thought to be important for kinase activity. Also, our analysis of the ORF3 protein sequence using the Prosite ProfileScan server, and analysis of ORF3 by another researcher using the Pfam and SMART databases (Mittenhuber, 2002) did not reveal any conserved kinase domains within the sequence. These findings suggest that unlike its homologs in *Bacillus*, the ORF3 protein product does not possess kinase activity.

3.1.2 Structural analysis of BldG and ORF3

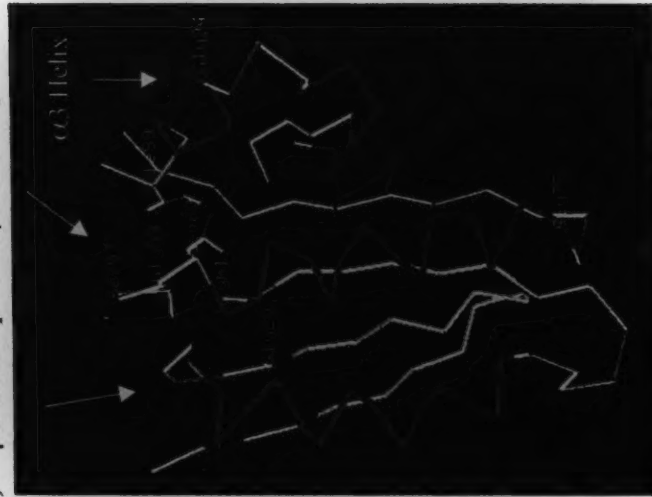
The solution structure of the *B. subtilis* SpoIIAA protein from has been determined (Kovacs *et al.*, 1998), and consists of 4 β -strands and 4 α -helices (Figure 3.1.1). To determine how the BldG structure compares to that of SpoIIAA, the SWISS-MODEL database was employed to model the BldG protein sequence using both SpoIIAA and RsbV proteins of *B. subtilis* as the template. The results shown in Figure 3.1.1 indicate that the predicted BldG structure consists of 3 β -strands, 2 long α -helices, and 3 short α -helices. The overall structure is very similar to the known structure of SpoIIAA, except in the C-terminal region where the β -strand in SpoIIAA is missing, and BldG contains an additional α -helix. In my hands, however, a C-terminal structure similar to that of the BldG predicted structure was determined for SpoIIAA using SWISS-MODEL, and since this predicted SpoIIAA structure was used as one of the modeling templates for BldG, it is unclear at this point whether the C-terminal difference between BldG and SpoIIAA is real, or if it simply represents a limitation of the modeling system. The 3-dimensional predicted structure of BldG is shown in Figure 3.1.3A, and again the overall shape and fold of the protein is very similar to that determined for SpoIIAA. Prominent features of the structure include the two long and parallel α -helices, and the two smaller α -helices at the C-terminus which stick out in a less regular arrangement called the panhandle (Aravind & Koonin, 2000). The serine residue (Ser57) which is the known phosphorylation site in SpoIIAA is located in the β 3- α 2 conserved loop, and the side chain is oriented away from the main body of the protein, which is similar to the crystal structure of the SpoIIAA protein from *B. sphaericus* (Seavers *et al.*, 2001). Figure 3.1.3B indicates the positions of the conserved

Figure 3.1.3 Predicted tertiary structure of BldG. The protein structure was predicted using the SWISS-MODEL database (<http://epasy.org/swissmod/SWISS-MODEL.html>) with SpoIIAA and RsbV as templates, and the PBD Viewer program (<http://us.epasy.org/spdbv>) was used to visualize the protein. α -helices are indicated in red, while β -strands are indicated in yellow, and non-structured amino acid sequences are indicated in white. (A) The location of the conserved serine residue (Ser57) which is phosphorylated in SpoIIAA is indicated, as well as the characteristic panhandle structure. (B) Amino acid residues in BldG which have been shown in SpoIIAA to be important for function are indicated, as well as the regions of the protein (β 2- α 1 loop, β 3- α 2 loop, α 3 helix) which are thought to be important for protein-protein interactions in SpoIIAA.

(A) Putative phosphorylation site (Ser57)



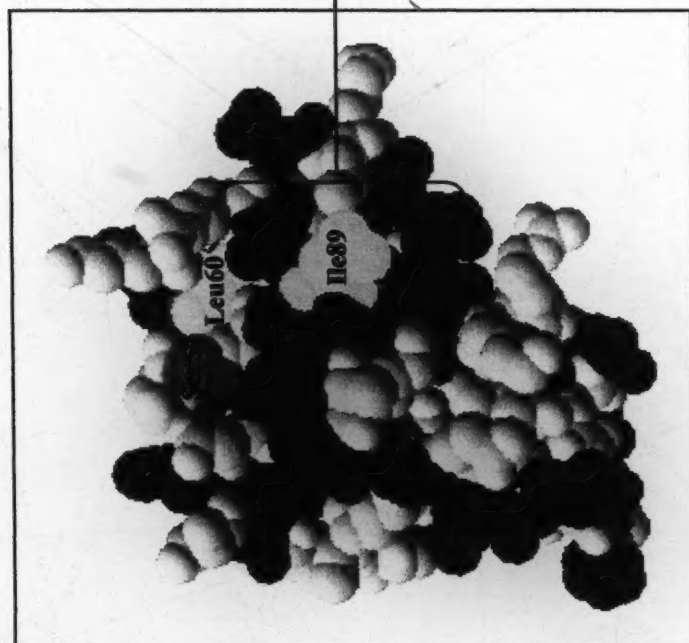
(B) $\beta 2$ - $\alpha 1$ Loop $\beta 3$ - $\alpha 2$ Loop



amino acid residues in BldG that have been shown in SpoIIAA to be important for protein function. Based on these mutational studies, it has been suggested that the $\beta 3$ - $\alpha 2$ loop, the $\beta 2$ - $\alpha 1$ loop, and the $\alpha 3$ helix of SpoIIAA are involved in protein-protein interactions with SpoIIAB or with the SpoIIIE phosphatase (Kovacs *et al.*, 1998). The high degree of sequence and structure conservation suggests that this might also apply to BldG when interacting with its binding partner. Recently it has been suggested that a contiguous patch of exposed hydrophobic residues on the surface of SpoIIAA in the vicinity of the phosphorylation site may also be important for protein-protein interactions (Clarkson *et al.*, 2001; Seavers *et al.*, 2001). Analysis of the BldG protein indicated a similar hydrophobic patch (see Fig. 3.1.4) present in the vicinity of Ser57, suggesting that residues in this patch may also be important for BldG function.

Modeling of the ORF3 protein structure was performed using SWISS-MODEL and using both RsbW and SpoIIAB of *B. subtilis* as the templates. Generally, all three proteins were predicted to have the same structure, with most of the variability occurring at the C-terminal region (Fig. 3.1.5). The ORF3 protein structure was predicted to contain 3 β -sheets and 2 α -helices, whereas RsbW was predicted to have 3 β -sheets and 3 α -helices, and SpoIIAB having 5 β -sheets and 4 α -helices. It has previously been suggested that the N-terminal region of SpoIIAB is responsible for complex formation with both its binding partners (SpoIIAA and σ^F) (Garsin *et al.*, 1998), and the high degree of structural conservation observed in the three proteins in this region may indicate a similar situation for both RsbW and ORF3.

Figure 3.1.4 Space-filled model of BldG. The model was drawn using the PBD Viewer program. Hydrophobic residues [C, I, L, M, F, W, Y, V, A, P, G; (Krystek Jr. *et al.*, 1995)] are indicated in blue, residues which are important for protein binding in SpoIIAA (Leu60, Ile89) are indicated in green, and the putative phosphorylation site (Ser57) is indicated in red. The location of the hydrophobic patch which may be important for BldG protein binding is indicated.



Hydrophobic
patch

Figure 3.1.5 Predicted protein structures of SpoIIAB and RsbW of *B. subtilis*, and of ORF3 of *S. coelicolor*. Structures were determined using the SWISS-MODEL database, with SpoIIAB and RsbW being used as templates for predicting the ORF3 structure. Models were visualized using the PBD Viewer program. α -helices are indicated in red, while β -strands are indicated in yellow, and non-structured amino acid sequences are indicated in white. The locations of the N and C terminal regions are shown.

SpoIIAB



ORF3



RsbW



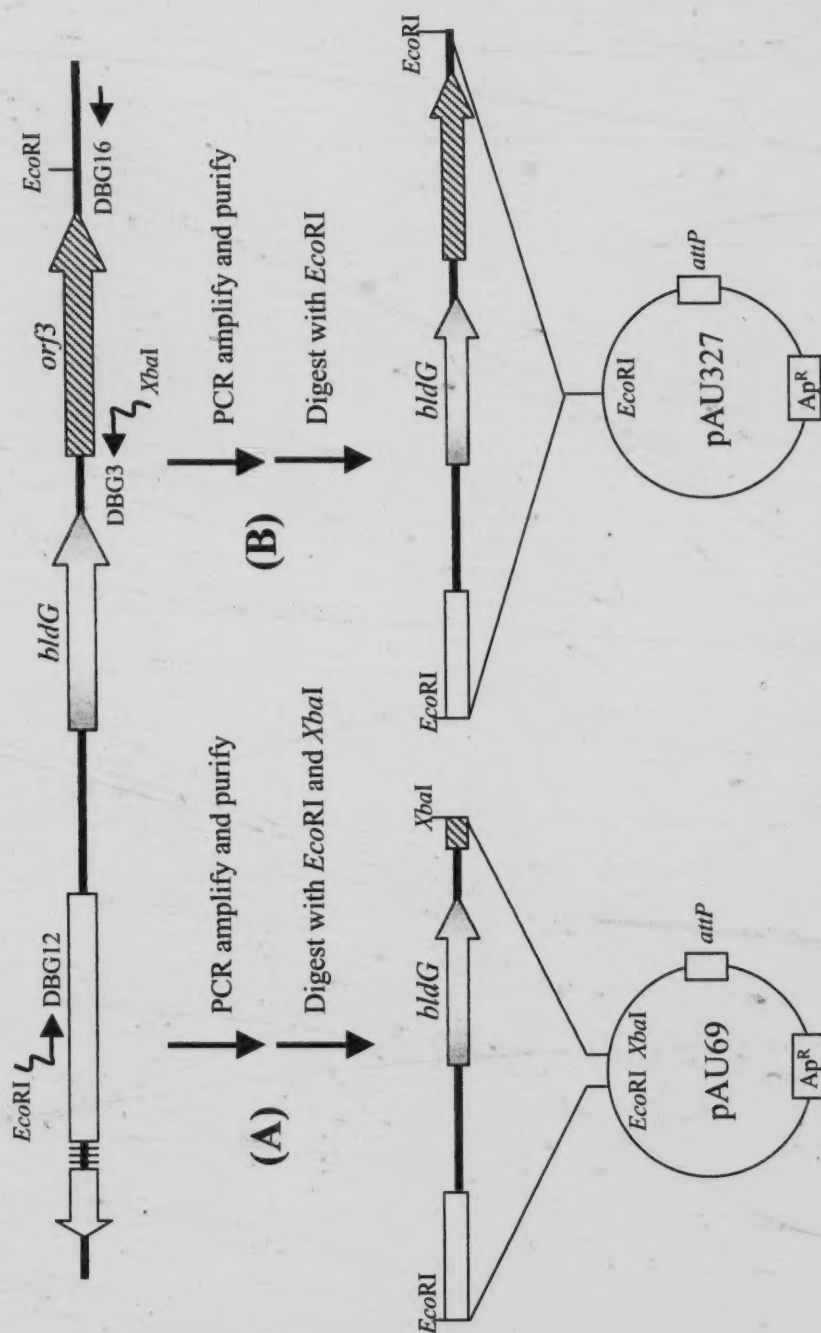
3.2 Mutational Studies of the *bldG* Locus

Mutational studies were performed in order to elucidate how BldG functions to control morphological and physiological development, and to determine whether ORF3 is involved in this process. The original *bldG* mutant strains C103, C107, C101J (Champness, 1988) and C536 that were isolated by Wendy Champness are all defective in the production of pigmented antibiotics and aerial hyphae when grown on R2YE medium. Sequencing of *bldG* in the C103 mutant strain had been performed by Jay Warawa, and we sought to determine the nature of the mutations in the other mutant strains. As well, we wanted to ascertain the phenotypic effects of a complete absence of BldG protein, and so an in-frame deletion allele for *bldG* was constructed and introduced into *S. coelicolor*. Site-directed mutagenesis was used to examine the effects of a S→A mutation at position 57 in BldG, and a deletion mutant of *orf3* was constructed in order to determine the phenotypic effects of such a mutation.

3.2.1 Complementation of *bldG* point mutants

To confirm that the *bldG107*, *bldG536* and the *bldG101J* point mutations isolated were located within the *bldG* coding region or promoter, the mutant strains were transformed with a plasmid that harbored the wild type copy of *bldG*. As shown in Figure 3.2.1, the complementation plasmid pAU69 was constructed by PCR amplification of a 937 bp fragment (DBG12-3) using pAU64 as the template and the primers DBG12 and DBG3 (see Table 2.5). The product was purified, digested with *EcoRI* and *XbaI*, and ligated into similarly-digested pSET152 to give pAU69. Three clones were isolated which contained insert of the correct size, and all were passaged

Figure 3.2.1 Strategy for construction of the complementation vectors pAU69 and pAU327. The *bldG* coding region is represented by the gray and white arrow, the *orf3* coding region is indicated by the hatched arrow, and the SCH5.13 coding region is indicated by the white arrow, with the direction of transcription of each indicated by the direction of the arrowheads. The location and binding direction of the primers used for PCR are indicated by the black arrows, with the curved ends representing non-homologous tails with engineered restriction sites, as indicated. (A) A 937 bp fragment containing the *bldG* coding region and promoter was amplified using the primers DBG12 and DBG3. The product was then digested with *EcoRI* and *XbaI* and ligated into similarly-digested pSET152 to give pAU69. (B) pAU327 was constructed by amplifying a 1489 bp fragment containing the *bldG* and *orf3* coding regions using the primers DBG12 and DBG16, and then digesting with *EcoRI* and ligating into similarly-digested pSET152. The Φ C31 *attP* site for integration into the *Streptomyces* chromosome and the apramycin-resistance gene (Ap^R) on each vector is indicated.



through *E. coli* ET12567 before being transformed into C107, C536 and C101J protoplasts. Apramycin-resistant transformants that arose for C107 and C536 all appeared wild type in their ability to produce pigmented antibiotics and aerial hyphae, whereas introduction of the plasmid vector alone (pSET152) had no effect, confirming that the point mutations were within the *bldG* coding region or promoter. On the other hand, pAU69 was not able to complement the mutant phenotype of C101J, which suggested that either the point mutation did not reside within *bldG*, or that this strain was a double mutant and could not be complemented by wild type *bldG* alone.

To determine whether the C101J strain had a second mutation in *orf3*, a new plasmid was constructed which harbored both the *bldG* and *orf3* coding regions. A 1489 bp fragment was amplified by PCR using Cosmid SCH5 as the template and the primers DBG12 and DBG16, and after purification, the product was digested with *EcoRI* and ligated into similarly-digested pSET152 to give pAU327 (see Figure 3.2.1). After sequencing to confirm that no PCR-induced mutations were present in the insert, pAU327 was passaged through ET12567, and transformed into C101J protoplasts. As with pAU69, apramycin-resistant transformants remained bald and did not produce pigmented antibiotics, indicating that the presence of both *bldG* and *orf3* wild type genes was not sufficient to complement the mutant phenotype.

3.2.2 Sequencing of *bldG* mutations

To map the location of the *bldG* mutation in C107 and C536 as well as to confirm the absence of a mutation in the *bldG* coding region of C101J, chromosomal DNA was isolated from each of the mutant strains, and was used as a template for PCR to amplify

Figure 3.2.2 Identification of point mutations in C107 and C536. Chromosomal DNA was isolated from each of the mutant strains, and *bldG* was amplified by PCR and sequenced. The nucleotide sequence of the coding strand is shown, with the nucleotide positions relative to the first nucleotide of the *bldG* start codon (designated +1) indicated on the right. The amino acid sequence of the protein is shown below the nucleotide sequence, with the stop codon indicated by (*). Point mutations identified in C107 and C536 are shown in bold, with the base change and amino acid change indicated above. The base insertion found in C107 is bolded and underlined and is shown above the nucleotide sequence. The location of the previously determined C103 point mutation is also shown.

CCGTGGACCTGTCCCTGTCGACCCGTACCGTCGGCGATCGTACGGTC +45
fM D L S L S T R T V G D R T V

GTCGAGGTCGGTGGCGAAATTGACGTATACACCGCGCCCAAGCTGCGT +93
V E V G G E I D V Y T A P K L R

GAGCAGCTGGTCGAGCTCGTGAACGACGGGAGTTTCCACCTCGTCGTC +141
E Q L V E L V N D G S F H L V V

bldG107 CTC AG (frameshift)

GACATGGAGGGCGTGGACTTCCTCGACTCCACAGGGCTCGGCGTGCTG +189
D M E G V D F L D S T G L G V L

GTGGGTGGACTGAAGCGGGTGCGTGCCCATGAGGGCTCGCTGCGGCTG +237
V G G L K R V R A H E G S L R L

bldG536, bldG103 T (K to stop)

GTCTGCAACCAGGAGCGCATTCTCAAGATCTTCCGTATCACCGGCCTC +285
V C N Q E R I L K I F R I T G L

ACCAAGGTGTTCCCCATTACACCTCGGTCGAGGAAGCGGTGGCGGCC +333
T K V F P I H T S V E E A V A A

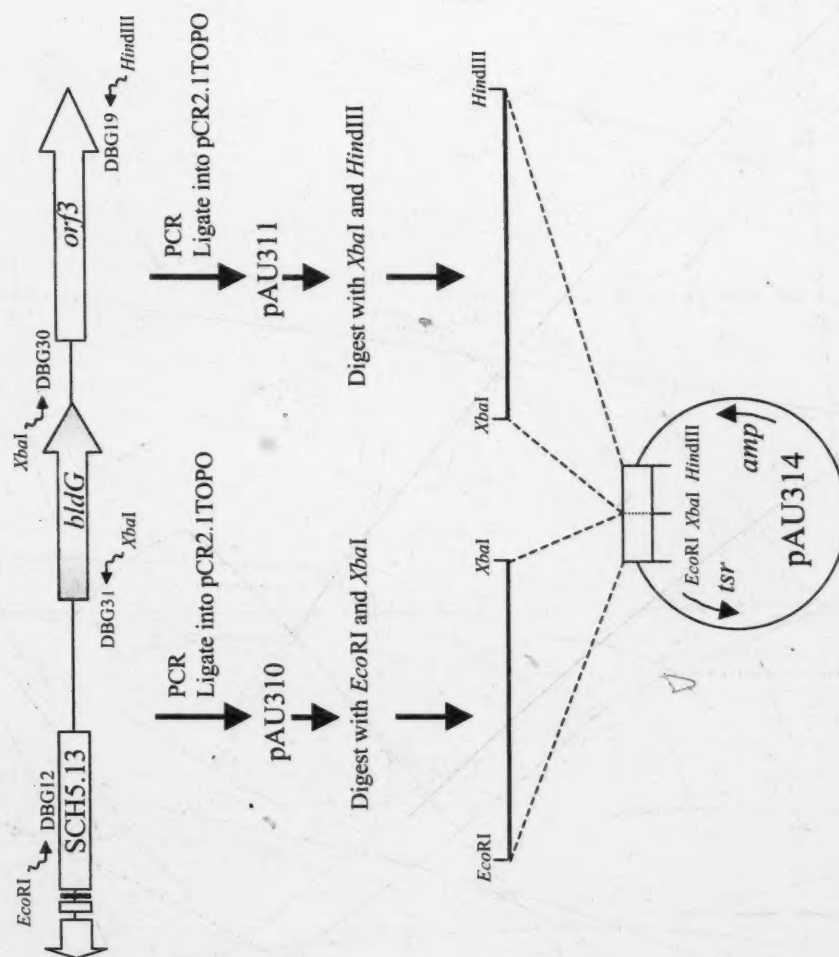
ACCGACTGA +342
T D *

bldG using the primers BKL65 or JWA5, and JWA6. The amplification products were then sequenced by the DNA Sequencing Service (Department of Biological Sciences) using the oligonucleotides BKL65 or JWA5, JWA6, DBG4, BKL92 and JWA1 as primers. As expected, *bldG101J* did not have a mutation in the *bldG* coding region or promoter region, and therefore was not studied further. The *bldG536* mutation was found to be identical to that of *bldG103* previously sequenced by Jay Warawa (Bignell *et al.*, 2000), and involves an A→T transition mutation which results in the introduction of a stop codon to generate a truncated 87 amino acid protein (Figure 3.2.2). In the case of *bldG107*, it was initially observed that this mutant contained two base substitutions at adjacent positions, resulting in an amino acid change of aspartate to glutamate at position 56, and of serine to alanine at position 57 of BldG. Further examination of the mutant sequence, however, revealed the presence of a third mutation, which introduces an extra T base just upstream of the base pair changes. This causes a frameshift mutation that alters the amino acid sequence of the protein starting at position 56, and truncates the protein at position 73.

3.2.3 Construction of a *bldG* in-frame deletion mutant

We next wanted to construct a deletion mutant of *bldG* to examine the phenotypic effects of a complete absence of BldG protein. At the time, BldG-specific antibodies were not yet available to look at BldG stability in the mutant strains, and it was possible that the point mutations that we had for *bldG* would simply reduce the BldG protein activity, but not completely abolish it. As a result, it was possible that the observed

Figure 3.2.3 Strategy for construction of the *bldG* in-frame deletion vector pAU314. A 527 bp fragment containing the 5' end of *bldG* was amplified by PCR using pAU69 as the template and the primers DBG12 and DBG31. The 3' end of *bldG*, including downstream sequence, was amplified by PCR as a 594 bp product using pAU66 as the template and the primers DBG30 and DBG19. The location and binding direction of each primer is indicated by the black arrows, with the curved ends representing non-homologous tails with engineered restriction sites, as indicated. Ligation of the gel-purified PCR products into pCR2.1TOPO generated pAU310 containing the 5' *bldG* region, and pAU311 containing the 3' *bldG* region. pAU310 was then digested with *EcoRI* and *XbaI* while pAU311 was digested with *HindIII* and *XbaI*, and the released fragments were ligated into *EcoRI* and *HindIII*-digested pHJL400 to give pAU314. The *bldG* coding region is represented by the gray and white arrow, while the SCH5.13 and *orf3* coding regions are represented by the white arrows, with the direction of transcription of each indicated by the direction of the arrowhead. The thiostrepton resistance gene (*tsr*) for plasmid selection in *Streptomyces*, and the ampicillin resistance gene (*amp*) for plasmid selection in *E. coli* are indicated by the black curved arrows.



phenotype of the mutant strains might not represent the phenotype of a strain in which BldG is not produced. The deletion of *bldG* was designed such that 84 of the 113 amino acids were removed, and it was an in-frame deletion to avoid any polar effects on the expression of the downstream *orf3* gene (see later). Figure 3.2.3 shows the procedure that was used for construction of the deletion. First, a 527 bp fragment containing the 5' end of *bldG* was amplified by PCR using pAU64 as the template and the primers DBG12 and DBG31. At the same time, a 594 bp fragment containing the 3' end of *bldG* was also amplified using pAU66 as the template and the primers DBG30 and DBG19. After purification, the two PCR products were ligated individually into pCR2.1TOPO to give pAU310 (containing the 5' *bldG* fragment) and pAU311 (containing the 3' *bldG* fragment). The plasmids were passaged through *E. coli* ET12567 and then digested with *EcoRI* and *XbaI* in the case of pAU310 to release a 517 bp fragment, and with *XbaI* and *HindIII* in the case of pAU311 to give a 584 bp fragment. The released fragments were ligated into the *EcoRI* and *HindIII* sites of pHJL400, and plasmid DNA isolated from ampicillin-resistant transformants was examined by digestion with *EcoRI* and *HindIII* to confirm the presence of insert, and sequencing was performed to confirm that the two fragments were ligated in-frame in the vector. One representative plasmid, designated pAU314, was passaged through *E. coli* ET12567, and was then transformed into *S. coelicolor* M145 protoplasts. Thiostrepton resistant transformants that arose would result from either integration of the plasmid into the chromosome by a single cross-over event at the *bldG* locus (Figure 3.2.4), or from the presence of freely replicating plasmid DNA, since the pHJL400 cloning vector used contains a *Streptomyces* origin of replication (Larson & Hershberger, 1986; see Table 2.3). Verification of plasmid integration in one

Figure 3.2.4 Integration of pAU314 into the *S. coelicolor* chromosome. (A) *S.*

coelicolor M145 protoplasts were transformed with pAU314, and thiostrepton resistant transformants were selected which contained the plasmid integrated into the chromosome at the *bldG* locus by a single cross-over event at either (1) or (2). The *bldG* coding region in the chromosome is represented by the gray and white arrow, while the deleted copy of *bldG* originating from the plasmid is represented by the smaller gray arrow with the hatched line down the centre. Chromosomal copies of *orf3* and SCH5.13 are indicated by the white arrows, while the plasmid copy of *orf3* is indicated by the hatched arrow, and the partial SCH5.13 sequence on the plasmid is indicated by the spotted box. The thiostrepton resistance gene (*tsr*) used for plasmid selection in *Streptomyces* is represented by the white box. Plasmid-specific DNA as well as *Streptomyces* intergenic DNA cloned into the plasmid are represented by the thin black line, whereas chromosomal-specific intergenic DNA is represented by the thick black line. The two different genetic arrangements of the chromosomal *bldG* locus resulting from the two possible cross-over events are shown in (B), as well as the sizes of two possible *orf3* containing *EcoRI* fragments.

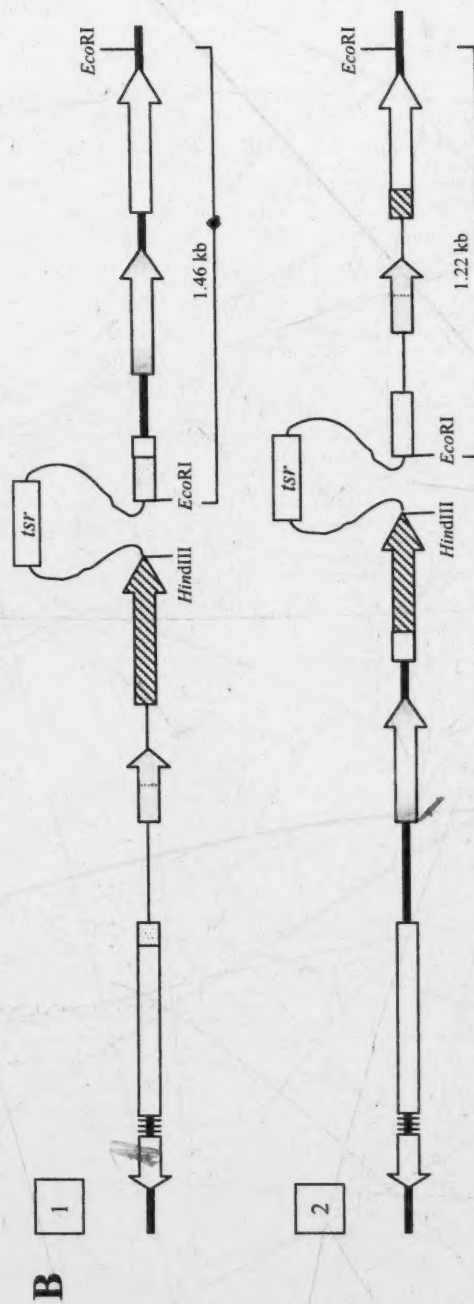
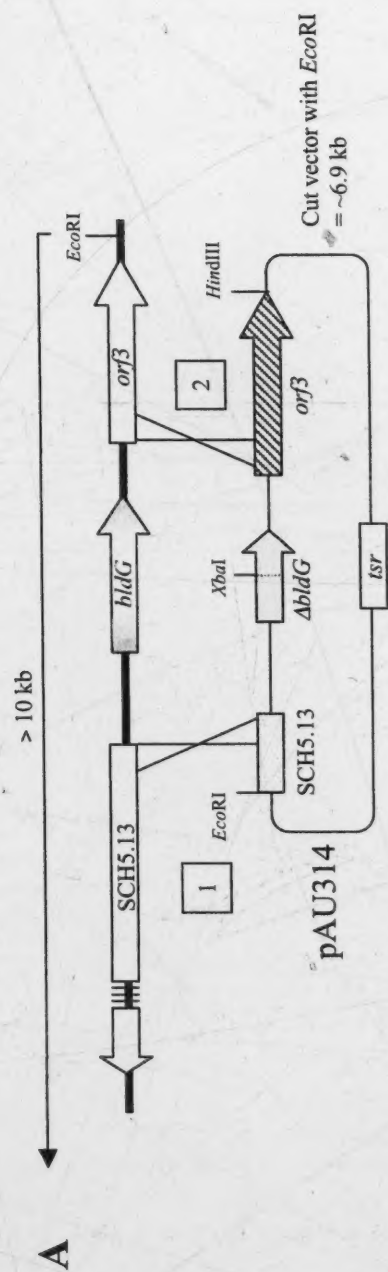
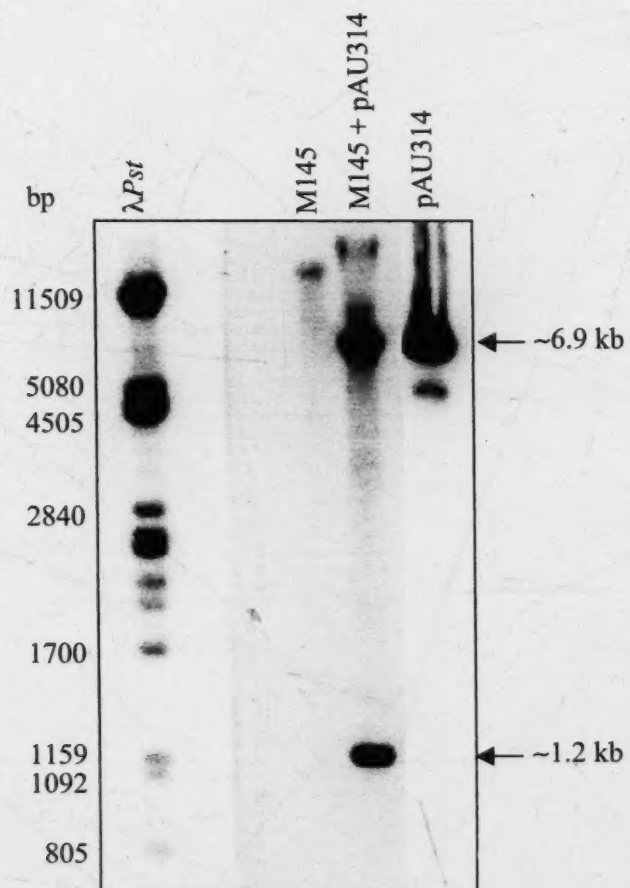


Figure 3.2.5 Verification of integration of pAU314 into the *S. coelicolor* chromosome. Chromosomal DNA isolated from *S. coelicolor* M145 and from a thiostrepton-resistant pAU314 transformant was digested with *Eco*RI and was electrophoresed along with *Eco*RI-digested pAU314 on a 1% agarose, 1× TBE gel. λ Pst DNA was included as the molecular weight marker. After transfer to Hybond-N membrane, the marker lane was cut off and probed with ^{32} P-random primer labeled λ Pst DNA while the remaining DNA was probed with a ^{32} P-random primer labeled PCR product internal to *orf3* and generated using the primers DBG8 and BKL83 (See Table 2.5). The presence of the ~1.2 kb *orf3*-containing fragment in the chromosomal DNA isolated from the thiostrepton resistant transformant verifies integration of the plasmid into the chromosome at the *bldG* locus (see Figure 3.2.4), whereas the ~6.9 kb *orf3*-containing fragment most likely represents plasmid that did not integrate into the chromosome. The sizes of the marker bands (in bp) are indicated.



thiostrepton-resistant transformant was by Southern analysis, in which chromosomal DNA was digested with *EcoRI* and then probed with a ^{32}P -labeled PCR product internal to *orf3* (Figure 3.2.5). To promote loss of the vector from this strain and replacement of the wild type gene with the in-frame deleted copy, unigenomic spores were serially diluted and plated non-selectively onto R2YE agar, and a single colony showing the typical *bldG* mutant phenotype was isolated and screened for loss of thiostrepton resistance. To confirm gene replacement in the chromosome, chromosomal DNA was digested with *EcoRI* and *BamHI*, and was then probed separately for *bldG* and *orf3* (Figure 3.2.6) by Southern analysis.

The mutant strain, designated $\Delta bldG$ 1DB, was unable to produce aerial hyphae or pigmented antibiotics on R2YE or minimal medium containing glucose, but could undergo sporulation when grown on minimal medium containing mannitol, which is consistent with the phenotype of the other *bldG* mutant strains. When the pAU69 plasmid (containing the wild type *bldG* ORF) was introduced into $\Delta bldG$ 1DB, aerial hyphae and antibiotic production were restored to wild type levels when grown on R2YE (Figure 3.2.7A), and analysis of the aerial hyphae by scanning electron microscopy revealed the presence of abundant spore chains (Figure 3.2.7B). On the other hand, introduction of the plasmid vector alone (pSET152) had no effect (Fig. 3.2.7A), indicating that the observed mutant phenotype of $\Delta bldG$ 1DB was due to the deletion of *bldG* and not due to polar effects on the downstream *orf3* gene.

Figure 3.2.6 Verification of gene replacement in $\Delta bldG$ 1DB. (A) Schematic diagram of chromosomal DNA isolated from M145 and $\Delta bldG$ 1DB. The DNA was digested with *Bam*HI and *Eco*RI to give either a ~1008 bp *orf3*-containing fragment in the case of M145, or a ~748 bp *orf3*-containing fragment in the case of $\Delta bldG$ 1DB, as indicated. The *bldG* coding region is shown as a gray and white arrow, while the *orf3* coding region is indicated by the hatched arrow, and the deleted copy of *bldG* is indicated by the gray arrow with the hatched line down the centre. (B) Digested chromosomal DNA was separated along with the marker (λ *Pst*) on a 1% agarose, 1× TBE gel, and was then transferred to Hybond-N membrane overnight. The membrane was probed with a ^{32} P-random primer labeled internal *orf3* PCR product generated using the primers DBG8 and BKL83, and which hybridized to the 1008 bp fragment from M145, and to the 748 bp fragment from $\Delta bldG$ 1DB, as shown. A ^{32} P-random primer labeled PCR product generated using the primers BKL63 and BKL64 and which corresponded to sequence deleted from *bldG* was found to hybridize to the same 1008 bp fragment from M145, but did not hybridize to any fragments from $\Delta bldG$ 1DB. The marker lane was cut off and probed with ^{32} P-random primer labeled λ *Pst* DNA in order to estimate band sizes.

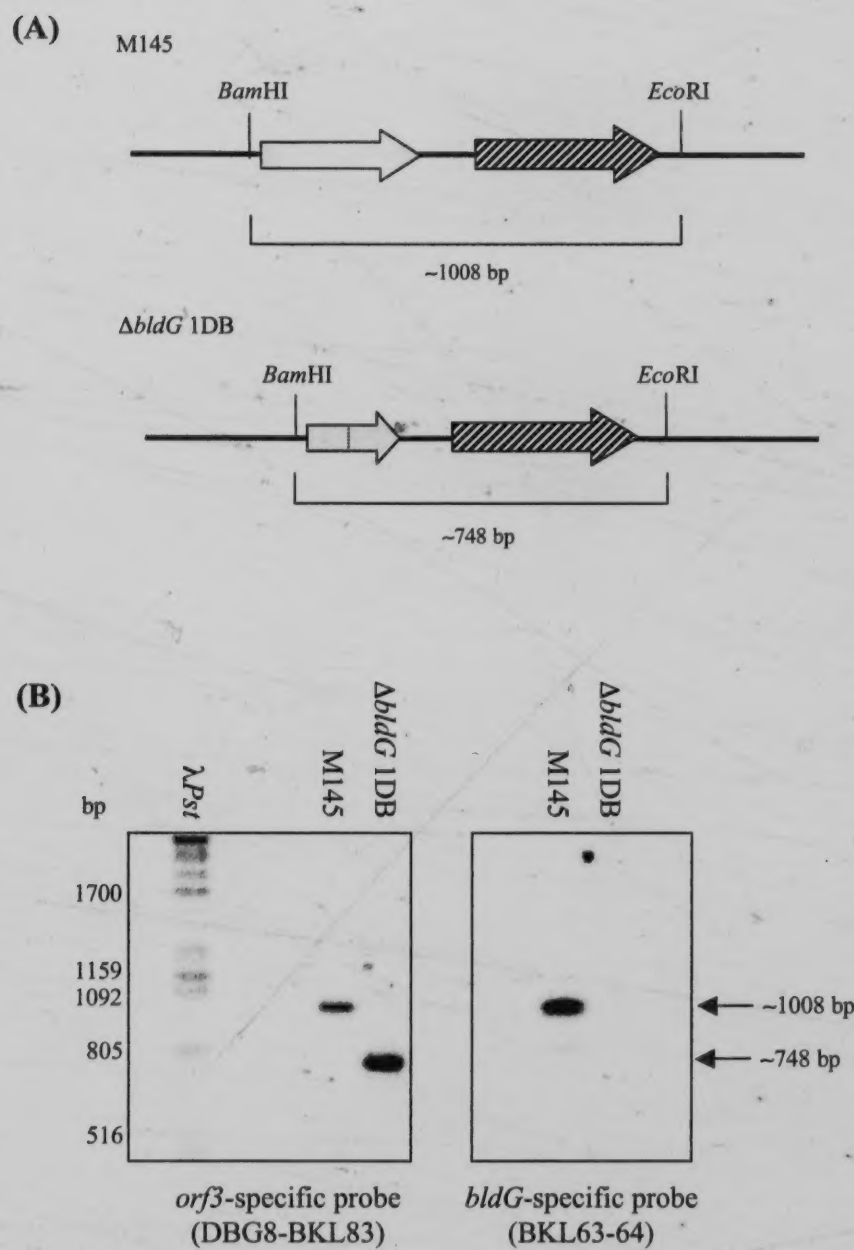


Figure 3.2.7 (A) Complementation of the $\Delta bldG$ 1DB mutant strain with a single copy of wild type *bldG* (pAU69) or the *bldGS57A* mutant gene (pAU325). The cloning vector alone (pSET152) is included as a negative control. (B) Scanning electron micrograph of aerial hyphae from the $\Delta bldG$ 1DB strain complemented with pAU69. The strain was grown on R2YE agar for one week before being subjected to scanning electron microscopy without further treatment. The magnification used (3500 $\times$) as well as the scaling (bar = 5 μ m) are indicated on the bottom of the picture.

(A)

bldG 1DB + pSET152

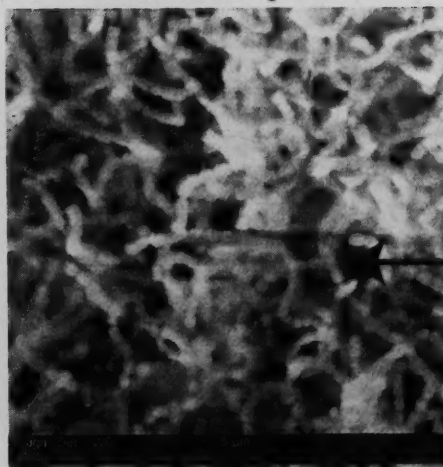
bldG 1DB + pAU69

bldG 1DB + pAU325



(B)

bldG 1DB + pAU69



Spore chains

3.2.4 Phenotypic effects of a *bldGS57A* mutation

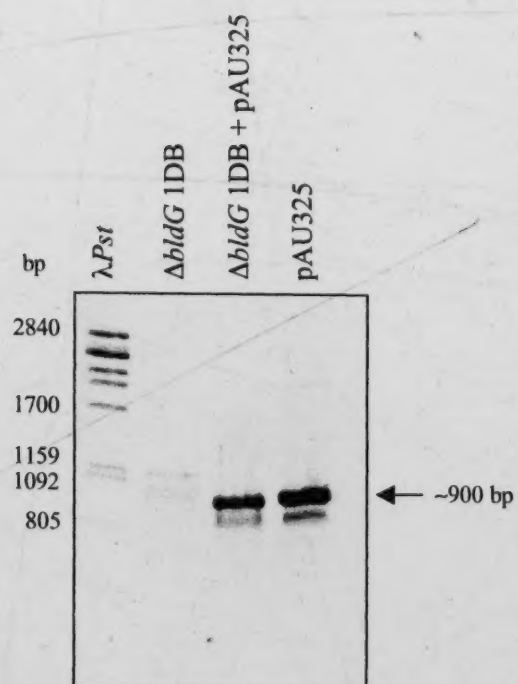
Alignment of BldG with other anti-anti-sigma factors had revealed that the serine residue at position 57 is conserved in all of the aligned proteins (see Figure 3.1.1), suggesting that this residue is important for proper function of anti-anti-sigma factors in general. Interestingly, this residue is the known phosphorylation site in SpoIIAA and RsbV (Najafi *et al.*, 1995; Yang *et al.*, 1996), and in other less-characterized anti-anti-sigma factor proteins (Beaucher *et al.*, 2002; Shi *et al.*, 1999), and phosphorylation is known to regulate the activity of the RsbV and SpoIIAA proteins (Alper *et al.*, 1994; Alper *et al.*, 1996; Dufour & Haldenwang, 1994; Duncan *et al.*, 1996; Magnin *et al.*, 1996). In the case of SpoIIAA, it has also been shown that mutation of this residue to an alanine residue (which cannot be phosphorylated) resulted in cells which lysed easily and could not undergo sporulation (Diederich *et al.*, 1994; Duncan *et al.*, 1996), suggesting that the regulation of SpoIIAA activity is necessary for proper growth and sporulation. We were therefore interested in determining the phenotypic effects of such a mutation in BldG.

Site-directed mutagenesis was first performed by amplifying a 460 bp fragment containing the entire *bldG* coding region using pAU64 as the template and the primers DBG35 and DBG22. The resulting PCR product was digested with *NdeI* and *EcoRI*, ligated into similarly-digested pAlter-Ex2, and tetracycline-resistant transformants were then screened for the presence of insert by digesting plasmid DNA with *NdeI* and *EcoRI*. A single representative, designated pAU319, was sequenced to ensure that no PCR-induced mutations were present, and was used for site-directed mutagenesis to change the TCC codon (serine) to a GCC codon (alanine) at position 57 using the oligonucleotide

DBG40, and to restore chloramphenicol resistance to the vector using the oligonucleotide WSC2. Clones isolated from chloramphenicol-resistant transformants were then sequenced to ensure that the desired mutation was present, and a single clone was designated pAU320.

To assess the phenotypic effect of the mutation, a 463 bp fragment was amplified using pAU320 as the template and the primers KC11 and KC12. The product was digested with *Bam*HI and *Xba*I and was ligated into similarly digested pAU69. A single clone, designated pAU325, was sequenced to ensure that the desired single site mutation was present and that no other mutations had been introduced, and the plasmid was transferred by conjugation from *E. coli* ET12567/pUZ8002 into the $\Delta bldG$ 1DB mutant strain. As shown in Figure 3.2.7A, the presence of pAU325 in $\Delta bldG$ 1DB resulted in the production of low levels of aerial hyphae and cell-associated red pigment within dense areas of growth, however the strain was unable to sporulate or produce blue pigmented antibiotics, even after prolonged incubation. To verify that the plasmid had not been lost from the $\Delta bldG$ 1DB strain, chromosomal DNA was isolated, and PCR was performed using the plasmid-specific primers JST7 and JST8 (see Table 2.5) in order to amplify the 463 bp *bldGS57A*-containing fragment. Figure 3.2.8 shows that a product was generated that was the same size as that generated using pAU325 as the template. This product was purified and sequenced, and was found to contain the *bldGS57A* coding sequence and promoter without any additional mutations present. Thus the inability of the *bldGS57A* mutant gene to restore the wild type phenotype to the $\Delta bldG$ 1DB mutant strain suggests that the Ser-57 residue is important for proper BldG function in *S. coelicolor*.

Figure 3.2.8 Amplification of the *bldGS57A* promoter and coding sequence from pAU325. PCR was performed using chromosomal DNA from $\Delta bldG$ 1DB containing pAU325 as the template and the primers JST7 and JST8, which anneal to sequence flanking the pAU325 polylinker. Reactions were also set up using chromosomal DNA from $\Delta bldG$ 1DB and pAU325 plasmid DNA as negative and positive controls, respectively. An aliquot of each reaction (5 μ l) was then electrophoresed on a 1% agarose, 1 $\times$ TBE gel along with λ *Pst* DNA included as the molecular weight marker, and the gel was stained with ethidium bromide in order to visualize the bands. The position of the ~900 bp amplification product is indicated.

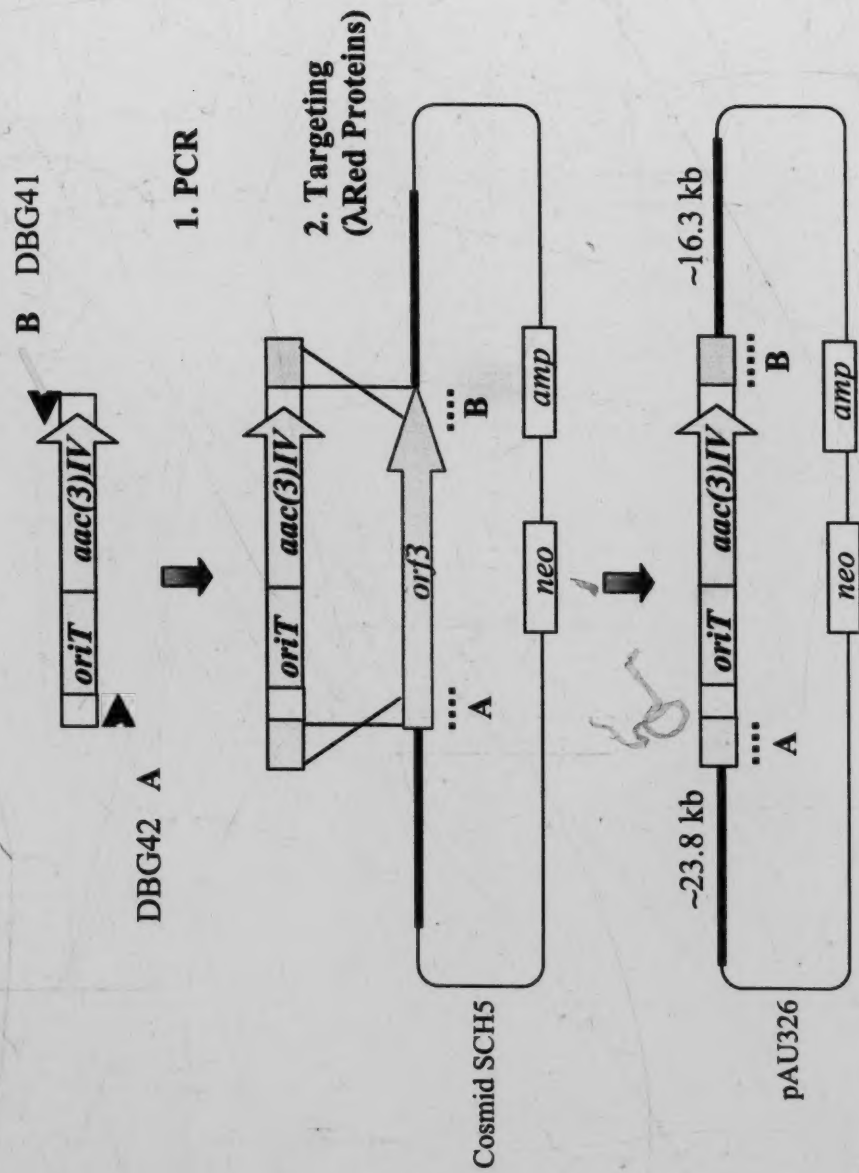


3.2.5 Deletion of *orf3* in *S. coelicolor*

In order to determine the phenotypic effects of an *orf3* mutation, attempts were made to disrupt *orf3* using an antibiotic resistance cassette, and to create an in-frame deletion of *orf3*. These methods, however, were unsuccessful, and so we then turned to a PCR targeting-based gene disruption protocol which was adapted to *S. coelicolor* (Gust *et al.*, 2003). This procedure, which is more efficient than those previously described to disrupt *Streptomyces* genes (Kieser *et al.*, 2000), involves recombination in *E. coli* between *S. coelicolor* DNA on a cosmid and a PCR-generated antibiotic cassette flanked at both ends by a short segment of nucleotides homologous to the desired region of the *S. coelicolor* DNA (Gust *et al.*, 2003). Recombination is mediated by the Red α (*exo*), Red β (*bet*) and the Red γ (*gam*) proteins of phage λ , which are encoded on the pIJ790 vector (see Table 2.3), and are expressed from an arabinose-inducible promoter in the *E. coli* strain that harbors the cosmid. The antibiotic cassette used [*aac(3)IV*] is selectable in both *E. coli* and *Streptomyces*, and an origin of transfer (*oriT*, RK2) is included in the disruption cassette to allow transfer of the PCR-targeted cosmid from *E. coli* to *Streptomyces* by conjugation. The advantage of using this procedure is that the disrupted gene in the cosmid is flanked by ~10-20 kb of sequence homologous to the *Streptomyces* chromosome, and as a result there is a greater chance of recombination events occurring between the cosmid and chromosomal DNA which lead to replacement of the wild type gene with the disrupted copy in the chromosome.

The procedure used to construct the PCR-targeted cosmid for deletion of *orf3* is described in Figure 3.2.9. PCR was performed using the primers DBG41 and DBG42, which contained 39 nucleotides of homology to sequence within *orf3* (see Table 2.5), and

Figure 3.2.9 Strategy for constructing the *orf3* deletion cosmid pAU326. A gene replacement cassette containing *aac(3)IV* (for apramycin resistance) and the *oriT* of RK2 (represented by the gray arrow and box, respectively) was amplified by PCR using the primers DBG41 and DBG42. The location and direction of the primer binding sites is indicated by the black arrows, with complementary sequence in the replacement cassette represented by the white boxes, and the colored lines (A and B) on the primers corresponding to 39 nt extensions which are homologous to sequence within the 5' and 3' regions of *orf3* (indicated by the hatched lines labeled A and B). The PCR product was used to transform *E. coli* BW25113/pIJ790 (expressing the λ Red proteins) containing cosmid SCH5 that harbours the *orf3* gene (indicated by the green and yellow arrow), and apramycin resistant transformants were selected. Gene replacement resulting from homologous recombination between the PCR product and the *Streptomyces* DNA in the cosmid was then verified by restriction digestion and by PCR. The *Streptomyces* DNA that flanks the wild type *orf3* gene in Cosmid SCH5 and the disrupted *orf3* gene in pAU326 is indicated by a bold black line, while cosmid-specific DNA is indicated by a thin black line. The kanamycin-resistance gene (*neo*) and the ampicillin resistance gene (*amp*) are shown as white boxes on the cosmid.



using a gel-purified 1384 bp *EcoRI/HindIII* disruption cassette [*aac(3)IV* + *oriT*] from pIJ773 as the template. The resulting product was purified using a PCR purification kit (Qiagen), and was then transformed into electrocompetent *E. coli* BW25113/pIJ790 cells harbouring Cosmid SCH5 (containing *orf3*) and containing the λ Red proteins (see Section 2.2.2). Transformants were plated onto LB agar containing ampicillin and kanamycin to select for the cosmid, and apramycin to select for replacement of *orf3* with the disruption cassette in the cosmid, and the cells were grown at 37 °C to promote loss of the temperature-sensitive pIJ790. Cosmid DNA from four transformants was analyzed by digestion with *SacI* and by PCR using the primers BKL62 and DBG16 to confirm gene replacement, and one clone, designated pAU326 (Figure 3.2.10), was then used to transform electrocompetent ET12567/pUZ8002. Deletion of *orf3* from the *Streptomyces* chromosome was accomplished by conjugating pAU326 from *E. coli* ET12567/pUZ8002 into *S. coelicolor* M145 spores, and then growing the exconjugants on MS agar in the presence of apramycin. Since the cosmid is unable to replicate in *Streptomyces*, apramycin-resistant exconjugants would arise as a result of either integration of the cosmid into the chromosome at the *orf3* locus, or from a double cross-over event resulting in replacement of the wild type *orf3* gene in the chromosome with the deleted copy (Figure 3.2.11). Exconjugants that arose were replica-plated onto MS agar without antibiotic, as well as onto Difco nutrient agar containing apramycin alone, or apramycin and kanamycin, in order to screen for strains that were apramycin resistant and kanamycin sensitive (and therefore had lost the cosmid). This was repeated 2 $\times$, and nine apramycin resistant, kanamycin sensitive exconjugants were chosen for further analysis.

Figure 3.2.10 Verification of gene replacement in pAU326. (A) Cosmid SCH5 and pAU326 were digested with *SacI* and analyzed by agarose gel electrophoresis. The loss of the doublet at ~1100 bp (indicated by τ) and the gain of the bands indicated by (*) verify that the *orf3* gene has been replaced by the disruption cassette.¹ (B) PCR was performed using Cosmid SCH5 and pAU326 as templates and the primers BKL62 and DBG16 to amplify *orf3*. The products were then analyzed by agarose gel electrophoresis. The change in product size from 802 to 1832 bp confirmed that gene replacement had occurred in pAU326.

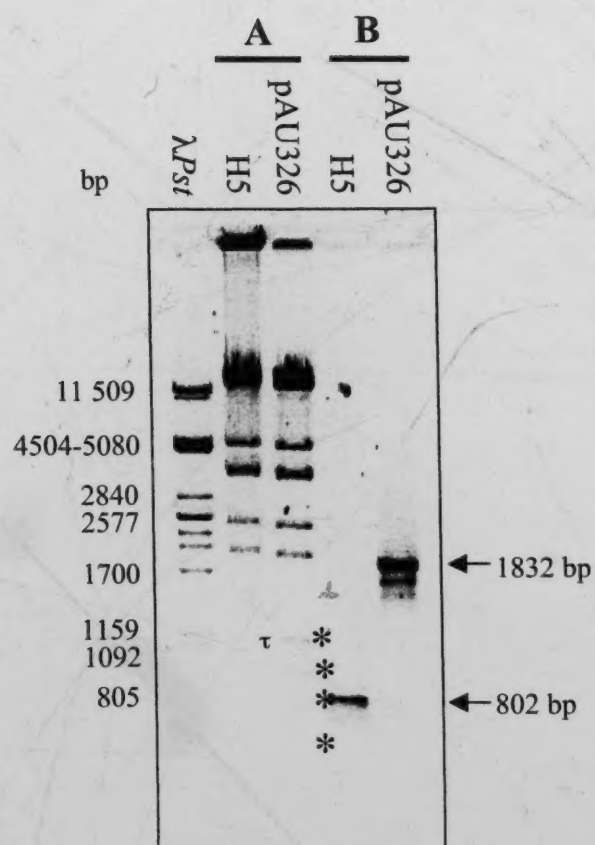
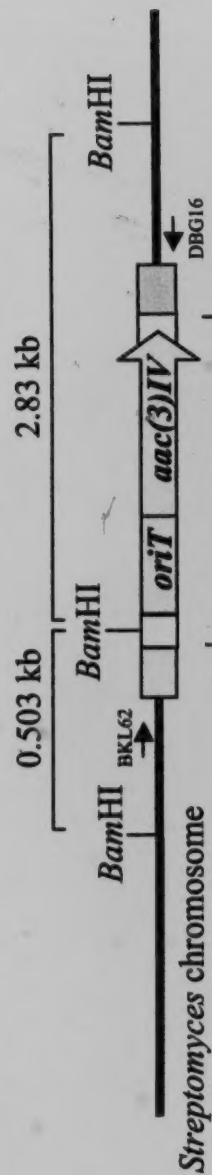
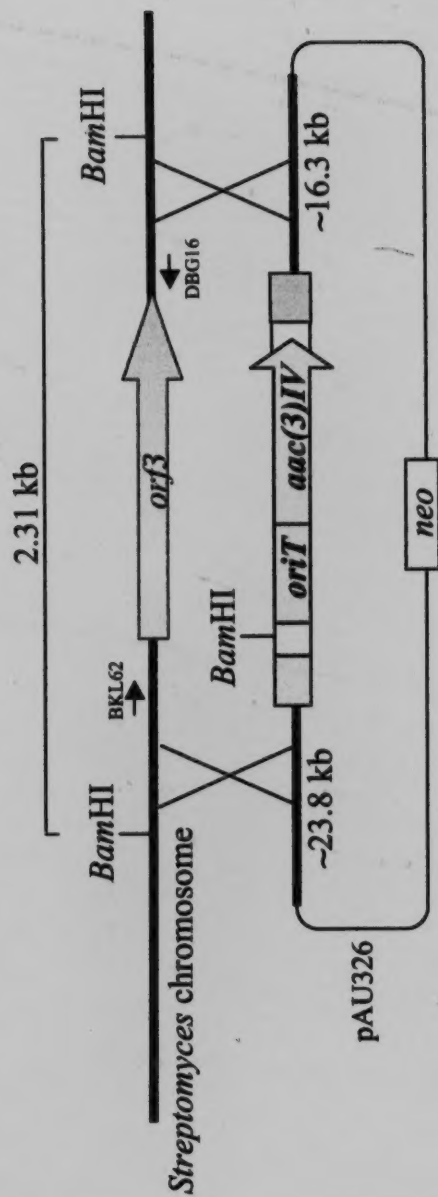


Figure 3.2.11 Replacement of the *orf3* gene in the *S. coelicolor* chromosome. The mutagenized cosmid pAU326 was conjugated into *S. coelicolor* M145 from *E. coli* ET12567/pUZ8002, and apramycin-resistant exconjugants were screened for kanamycin sensitivity, indicating that a double cross-over event had resulted in loss of the cosmid and replacement of the wild type *orf3* gene (indicated by the yellow and green arrow) with the deleted copy of *orf3* (represented by the *acc(3)IV + oriT* gray arrow and box surrounded by the yellow, green and white boxes). Homologous sequences on the cosmid and chromosome used for recombination are indicated as a bold black line, whereas cosmid-specific DNA is shown as a thin black line. The *Bam*HI sites and the fragment sizes resulting from *Bam*HI digestion used to verify gene replacement in the chromosome by Southern analysis are indicated, as well as the location and binding direction of the BKL62 and DBG16 primers (represented by black arrows) used to PCR-amplify the *orf3* gene in the wild type and mutant strains. The kanamycin resistance gene on the cosmid (*neo*) is indicated by the white box.

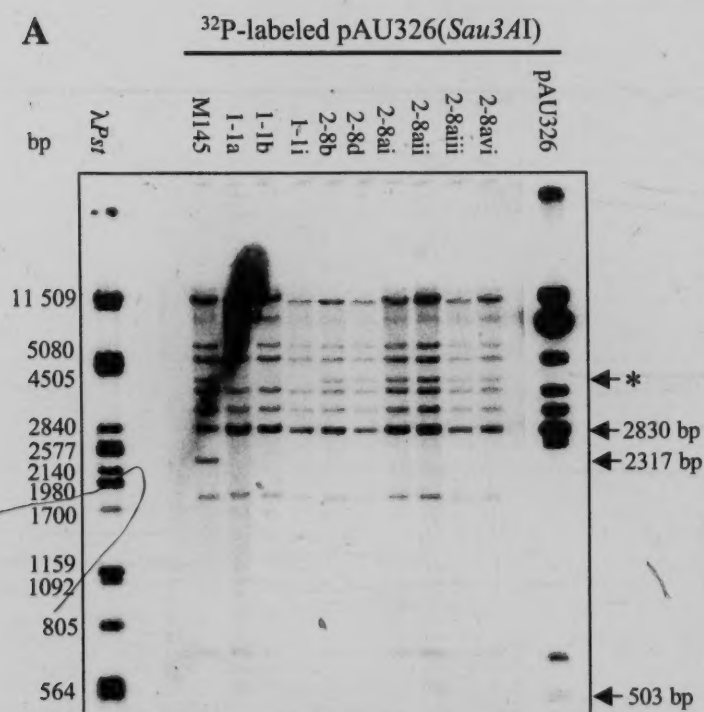
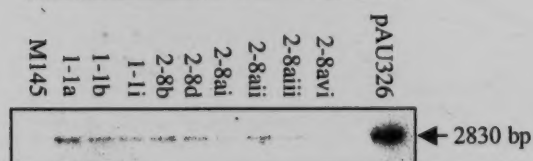
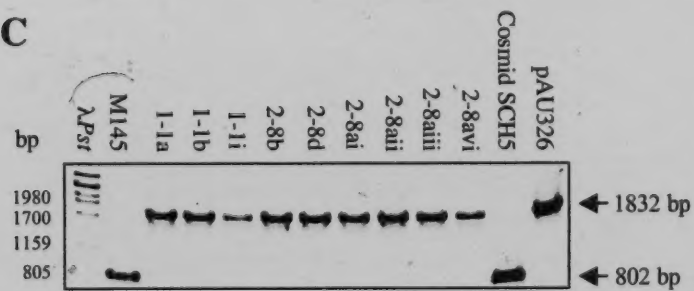


354 bp of *ori3* replaced with 1384 bp disruption cassette

To verify that gene replacement had taken place in the nine putative *orf3* mutants, Southern analysis was performed in which chromosomal DNA isolated from M145 and the nine mutant strains was digested with *Bam*HI, and probed with ³²P-random primer labeled pAU326 that had been digested with *Sau*3AI. Figure 3.2.11 shows that if gene replacement occurred in the chromosome, there would be loss of a 2.31 kb *Bam*HI fragment, and the appearance of a 2.83 kb and a 0.503 kb *Bam*HI fragment. The results, shown in Figure 3.2.12(A), confirm that in all nine mutant strains, replacement of the wild type *orf3* gene by the mutant copy had taken place in the chromosome. Interestingly, a ~4 kb band that was detected in the wild type M145 lane was absent from three of the nine mutant lanes and from the pAU326 mutant cosmid lane. This band was also missing from *Bam*HI-digested cosmid SCH5 (not shown), and its absence was not attributed to partial digestion of the DNA, as repeated digestions using higher amounts of enzyme and lower amounts of DNA did not change the results (not shown). Since analysis of the previously determined M145 DNA sequence in cosmid SCH5 (see http://www.sanger.ac.uk/Projects/S_coelicolor/) also failed to detect the presence of such a band, it is possible that the cloned 40.54 kb *S. coelicolor* DNA in this cosmid contains a mutation that results in loss of a *Bam*HI site. This would explain why only some of the mutant strains are missing the ~4 kb band, as inclusion of this mutation into the chromosome would depend on where the crossover event between the cosmid and the chromosome took place. Figure 3.2.12(B) was generated by probing the same DNA samples from Figure 3.2.12(A) with the [*aac(3)IV+oriT*] disruption cassette, and these results confirm that the observed apramycin resistance of the mutant strains is due to the presence of the disruption cassette, and not due to spontaneous apramycin-resistance.



Figure 3.2.12 Verification of *orf3* gene replacement in *S. coelicolor*. Chromosomal DNA isolated from *S. coelicolor* M145 and the nine putative *orf3* mutants was digested with *Bam*HI and was separated along with λ Pst on a 1% agarose gel. pAU326 digested with *Bam*HI was also included as a positive control. After transfer to membrane, the marker lane was cut off and was probed with ^{32} P-random primer labeled λ Pst DNA, while the chromosomal DNA samples were probed with either ^{32} P-random primer labeled pAU326 that had been digested with *Sau*3AI (A) or with the ^{32} P-labeled [*aac(3)IV+oriT*] disruption cassette (B). (A) The sizes of the molecular weight marker bands are indicated (in bp), as well as the position and size of the bands that were used to verify gene replacement in the mutant strains. (*) denotes the *Bam*HI fragment that is missing in three of the nine *orf3* mutant strains and in the pAU326 mutant cosmid. (C) The *orf3* gene in M145 and the nine putative *orf3* mutants was amplified by PCR using BKL62 and DBG16 as primers. Control templates that were used included Cosmid SCH5 and pAU326. The products were analyzed by agarose gel electrophoresis using λ Pst as the molecular weight marker, and the marker band sizes as well as the sizes of the observed product bands are indicated.

A**B**³²P-labeled [*aac*(3)*IV*+*oriT*] cassette**C**

Gene replacement was also verified by PCR, where the *orf3* gene was amplified from the chromosomal DNA of wild type and mutant strains using the primers BKL62 and DBG16, which would generate a ~802 bp product in the case of the wild type gene, and a ~1832 bp product in the case of the mutant gene. As shown in Figure 3.2.12(C), the observed increase in size of the product from the mutant strains compared to that from the wild type strain correlates with the Southern data in suggesting that allelic replacement has occurred in these strains.

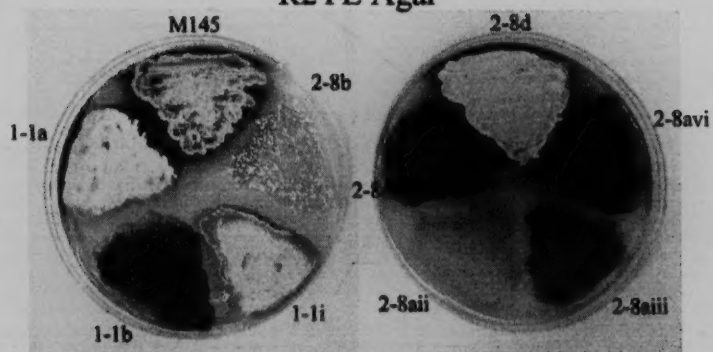
3.2.6 Phenotypes of *orf3* deletion mutants

The *orf3* mutation was designed such that 117 of the 143 amino acids would be deleted from the ORF3 protein. To determine the phenotypic effect of the deletion of *orf3*, mutant strains were plated along with the M145 wild type strain onto R2YE, MS agar, ISP-4 agar, minimal medium + glucose and minimal medium + mannitol, and were grown at 30 °C. Figure 3.2.13 shows that a variety of phenotypes were observed for the different mutant strains when grown on R2YE, MS agar and ISP-4 medium for 72 hours. The following observations were made for each mutant strain.

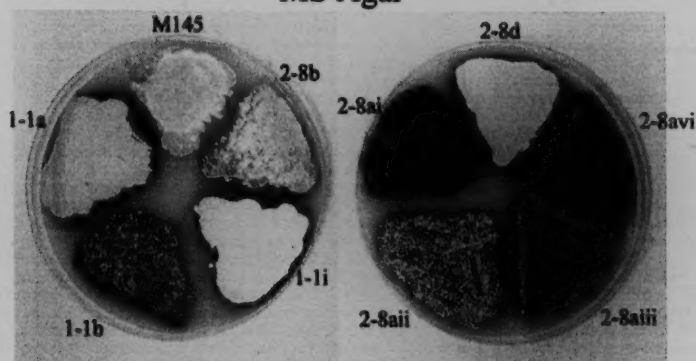
(a) 1-1a, 1-1i, 2-8d. On R2YE agar, all of these mutants grew at a rate similar to that of the wild type strain and produced aerial hyphae and pigmented antibiotics, although the production of the blue pigmented actinorhodin was significantly delayed in the case of 1-1a and 1-1i, which showed levels similar to M145 only after about 14 days incubation. On MS agar, 1-1a, 1-1i and 2-8d showed similar levels of aerial hyphae and accelerated blue pigment production compared to the wild type strain, whereas on ISP-4 medium, production of blue pigment and aerial hyphae was significantly delayed in all

Figure 3.2.13 Growth of *S. coelicolor* M145 and the nine *orf3* deletion mutants on R2YE, MS agar and ISP-4 agar. The *orf3* deletion mutants are designated 1-1a, 1-1b, 1-1i, 2-8b, 2-8d, 2-8ai, 2-8aii, 2-8aiii and 2-8avi. The plates were incubated at 30 °C for 72 hours.

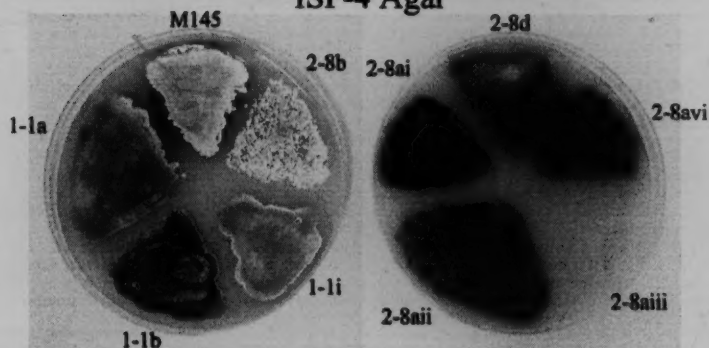
R2YE Agar



MS Agar



ISP-4 Agar



strains, with 2-8d being the most severely delayed of the three in terms of aerial hyphae formation.

(b) 1-1b. The 1-1b mutant strain produced significantly less aerial hyphae than M145 on all three types of media, with the least amount being observed on ISP-4 medium. This strain was also unable to produce any blue pigment on any medium, even after prolonged incubation. Interestingly, the aerial hyphae produced by this strain often appeared pink in color instead of the typical white color, suggesting that unlike in M145, production of the cell-associated red pigment may be occurring to some extent within the aerial hyphae as opposed to being localized exclusively to the substrate hyphae.

(c) 2-8b. Aerial hyphae formation and pigmented antibiotic production by the 2-8b mutant strain were both reduced on R2YE and ISP-4, with only small patches of aerial hyphae and minimal amounts of pigment being observed even after 14 days of incubation. As well, the growth on ISP-4 was also found to be significantly less than that observed for the wild type strain. On MS agar, on the other hand, aerial hyphae production was only slightly delayed compared to M145, and blue pigment production was actually accelerated.

(d) 2-8ai, 2-8aaii, 2-8aiii, 2-8avi. These mutant strains all had a bald appearance after 72 hours of growth on R2YE, and produced varying levels of the red cell-associated pigment and no blue pigment. All but one of the mutant strains (2-8aiii) produced aerial hyphae and blue pigment after prolonged incubation (14 days), although the levels of each remained lower than that observed for the wild type strain. On MS agar, all strains displayed accelerated blue and red pigment production, however aerial hyphae formation was again significantly delayed after 72 hours incubation, and only 2-8aaii was able to

produce wild type levels of aerial hyphae after prolonged incubation. On ISP-4 plates incubated for 72 hours, 2-8ai, 2-8aii, 2-8avi produced reddish-burgundy cell-associated pigment and no blue pigment, and were severely delayed in the production of aerial hyphae. Blue pigment production was similar to wild type levels after 14 days incubation, however aerial hyphae formation remained low. Interestingly, the 2-8aiii strain was unable to grow at all on ISP-4 medium, even after prolonged incubation.

The growth of the mutant strains was also examined on minimal medium + glucose and minimal medium + mannitol. Generally, the amount of aerial hyphae produced by the strains was less than that observed for M145 after incubation for up to 14 days, and in many cases, aerial hyphae formation did not occur at all on minimal medium + mannitol. Production of the red cell-associated pigment was also reduced, with the exception of 1-1b and 2-8ai, which produced similar levels of red pigment to M145. The production of spores by the mutant strains grown on the various media was not investigated.

3.2.7 Complementation of *orf3* mutants

The fact that a variety of phenotypes were observed on different media for the nine $\Delta orf3$ mutants suggested that some (or all) of these mutants may have acquired secondary site mutations in other genes. A similar phenomenon has been observed in *B. subtilis*, where deletion of the *spoIIAB* anti-sigma factor gene was shown to be toxic to the cell under conditions which induce sporulation, and often resulted in the acquisition of secondary site mutations in the corresponding sigma factor (σ^F) (Schmidt *et al.*, 1990). Moreover, a *spoIIAB* deletion mutant could only be obtained in a background strain in

which a mutation in σ^F partially suppressed the toxic effects of the *spollAB* deletion without affecting sporulation, or in a strain that was never allowed to enter stationary phase before being subjected to sporulation conditions (Coppolecchia *et al.*, 1991). In *S. coelicolor*, disruption of the RsrA and the RsuA anti-sigma factors has also led to the isolation of spontaneous suppressor mutants that were found to contain mutations in the target sigma factor gene, and in both cases, reversion to the disruption mutant phenotype occurred when the wild type sigma factor gene was introduced into the suppressor mutants on a plasmid (Gehring *et al.*, 2001; Paget *et al.*, 2001b). Presumably, these suppressor mutations served to reduce the activity of the target sigma factor in order to avoid any toxic effects under conditions where it is normally inactivated.

If the $\Delta orf3$ mutants contained secondary site mutations, then introduction of the wild type *orf3* gene alone would not restore the mutants to the wild type phenotype. Complementation experiments were therefore performed in which the *orf3* gene was introduced into each of the mutant strains on an *att*-site integrative plasmid. The first set of complementation experiments carried out involved the use of the pAU323 vector in which expression of *orf3* was under control of the thiostrepton-inducible *ptipA* promoter. To construct this vector, the *orf3* coding region was PCR-amplified using pAU66 as the template and the primers DBG33 and DBG34 (see Table 2.5). After purification, the PCR product was digested with *NdeI* and *XbaI* and ligated into similarly-digested pIJ6902. Clones were then screened for the presence of insert by digestion with *NdeI* and *XbaI*, and one clone, designated pAU317, was sequenced to ensure that no mutations were introduced during PCR. Since the $\Delta orf3$ mutants were originally created using the apramycin-resistance cassette, a new selection marker was needed to replace the

apramycin resistance marker on pAU317. This was accomplished by digesting pAU317 with *Bam*HI and *Bgl*II, ligating the cohesive ends of the plasmid together (in order to remove a portion of the polylinker containing a *Sac*I site), and then digesting this new plasmid with *Sac*I to release the apramycin resistance gene. The *Sac*I-digested plasmid was then ligated with a gel-purified ~2 kb *Sac*I fragment from pT06 (see Table 2.3) containing the spectinomycin resistance gene, and the new plasmid was designated pAU323. As a control for the complementation experiments, a pIJ6902 derivative (designated pAU322) was constructed in which the apramycin resistance cassette was replaced with the spectinomycin resistance gene in the same way that was described for construction of pAU323.

The pAU322 and pAU323 plasmids were introduced into each of the nine $\Delta orf3$ mutant strains by conjugation, and the phenotypic effects were assessed after growing exconjugants onto R2YE plates in the presence and absence of thiostrepton. Unfortunately, the results were difficult to interpret and were largely inconclusive for most of the strains, since it became obvious that the presence of thiostrepton in the medium affected the growth and appearance of many of the mutant strains in a manner that was independent of whether the wild type *orf3* gene was present or not. As well, since the *orf3* gene in pAU323 was being expressed from a stronger promoter than the native *orf3* promoter, the level of ORF3 protein present in the strains would not represent what was normally found in *S. coelicolor*, which in turn could affect whether complementation was observed or not.

A new strategy was therefore undertaken in which the mutant strains were complemented with the wild type *orf3* gene expressed from the upstream *bldG* promoter.

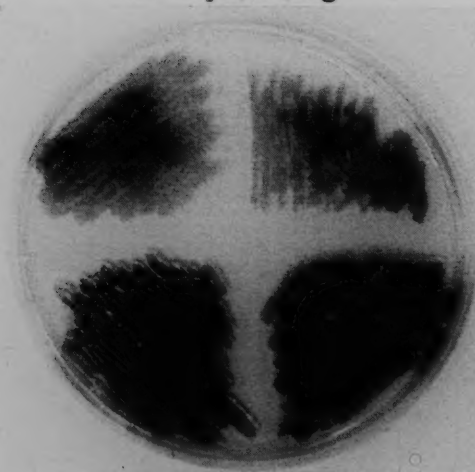
The plasmid pAU324 was constructed by digesting pAU314 with *Hind*III, blunting the *Hind*III end, and then digesting with *Eco*RI to release a 1097 bp fragment containing the *bldG* promoter, the deleted *bldG* coding region and the *orf3* coding region (see Section 3.2.3). At the same time the *att* site-integrating plasmid pSET Ω , which contains the spectinomycin resistance cassette for plasmid selection (see Table 2.3), was digested with *Xba*I, blunted, and then digested with *Eco*RI. Reactions were then set up in which the *orf3*-containing fragment was ligated into the digested pSET Ω , and clones were screened for the presence of insert by restriction digestion with *Eco*RI and *Bam*HI. A single clone, designated pAU324, was then introduced into each of the mutant strains by conjugation from *E. coli* ET12567/pUZ8002. As a control, the pSET Ω cloning vector was also conjugated into each strain, and resulting pSET Ω and pAU324 exconjugants were grown on R2YE, MS agar and ISP-4 medium in order to assess the phenotypic effects of the plasmids.

For the majority of the mutants, it was found that introduction of the wild type *orf3* gene had no effect on the growth of the strains on the various media. This includes the 2-8aiii strain, which was still unable to grow on ISP-4 medium even with the *orf3* gene present (data not shown). In the case of the 2-8d strain, accelerated growth and blue pigment production was observed with the *orf3*-complemented strain compared to the control when grown on R2YE medium for 48 hours (Figure 3.2.14). On the other hand, the delay in morphological differentiation previously observed by this strain on ISP-4 medium compared to the wild type strain (Figure 3.2.13) was not rectified by the presence of *orf3* when grown for a period of time that allowed for normal development of this strain on MS agar (Figure 3.2.14). For the 2-8avi strain, the presence of *orf3* actually

Figure 3.2.14 Growth of the $\Delta orf3$ 2-8d and 2-8avi strains complemented with pSET Ω (negative control) or pAU324 (containing *orf3*) on MS agar, ISP-4 medium and/or R2YE. The 2-8d strains were grown at 30 °C for 24 hours on R2YE, whereas the rest were grown for 72 hours.

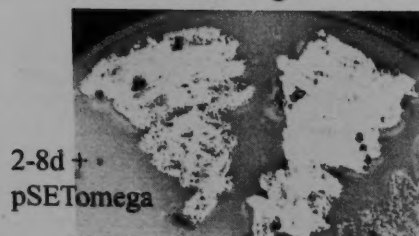
R2YE

2-8d + pSETomega



2-8d + pAU324

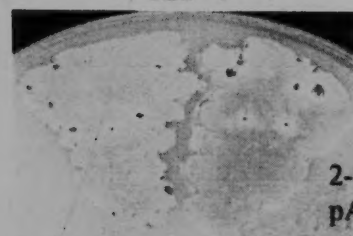
MS Agar



2-8d +
pSETomega

2-8d + pAU324

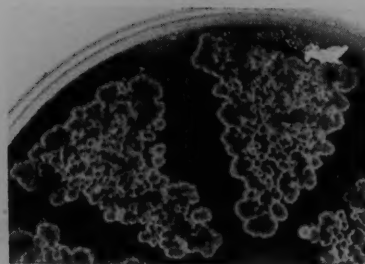
ISP-4



2-8d +
pAU324

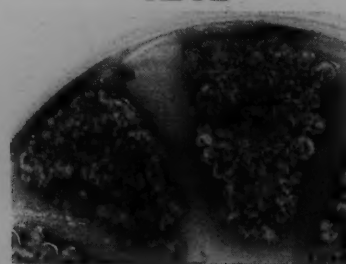
2-8d + pSETomega

R2YE



2-8avi + pSETomega

R2YE



2-8avi + pAU324

resulted in delayed aerial hyphae formation and antibiotic production compared to the control strain on R2YE medium (Figure 3.2.14), however growth on MS and ISP-4 medium was no different between the control and the *orf3*-complemented strains, even though the 2-8avi mutant had previously showed delayed development on these media after similar incubation times (Figure 3.2.13).

The results of the complementation experiments, therefore, suggest that the majority of the $\Delta orf3$ mutants isolated most likely contain secondary site mutations in one or more genes, which are in turn responsible for the observed phenotypes of the strains. While the presence of the wild type *orf3* gene affected the development of the 2-8d and the 2-8avi strains on R2YE medium, the inability of the *orf3* gene to complement the mutant phenotypes on other growth media suggests that these mutants may also contain secondary site mutations in other genes. Whether *orf3* plays a key role in the regulation of development in *S. coelicolor*, therefore, remains to be elucidated.

3.3 Transcriptional Analysis of the *bldG* Locus

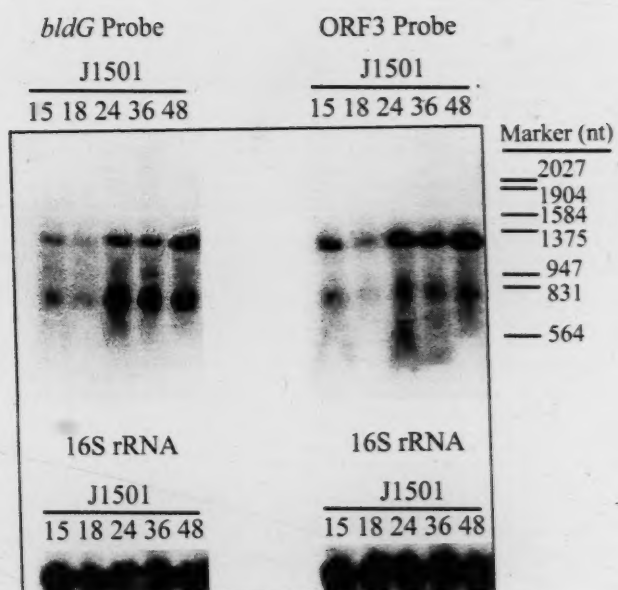
In addition to mutational studies, our attempt to characterize the *bldG* locus involved examining the regulation of *bldG* and *orf3* gene expression during development. Typically, genes encoding anti-anti-sigma factor and anti-sigma factors are co-transcribed as a polycistronic transcript, and experiments were performed to determine whether this was also the case for *bldG* and *orf3*. As well, we were interested in examining the timing of expression of *bldG* and *orf3*, and determining the locations of transcription start sites.

Finally, the effect of environmental stresses such as osmotic (salt) shock on gene expression was also investigated.

3.3.1 Northern analysis of *bldG* and *orf3*

Northern analysis was performed using RNA isolated from *S. coelicolor* J1501 at various times following inoculation in order to determine the size of the *bldG* and *orf3* transcripts and to examine the timing of expression during development. The probe for *bldG* was a random primer labeled, 207 bp fragment homologous to internal *bldG* sequence, and was generated by PCR using pAU64 as the template and the primers BKL63 and BKL64. In the case of *orf3*, the probe was a random primer labeled, 428 bp PCR product internal to *orf3*, and was generated using pAU66 as the template and the primers DBG8 and BKL83. The experiments were performed at least twice using RNA from three different time courses, and representative results are shown in Figure 3.3.1. The *bldG*-specific probe was found to hybridize to two RNA transcripts with sizes of about 600-700 nt and 1100-1200 nt. The size of the smaller transcript is consistent with a *bldG* monocistronic transcript, whereas the size of the larger transcript agrees with that expected for a *bldG-orf3* polycistronic transcript. When the *orf3*-specific probe was used in hybridization experiments, it detected only one transcript, the size of which corresponded to the larger *bldG* transcript. The transcripts were found to be present at low levels during vegetative growth (15 and 18 hours post-inoculation), and were found to increase starting at 24 hours post-inoculation, which correlates with the appearance of both aerial hyphae and pigmented antibiotics. When the same blots were probed with an oligonucleotide specific for 16S rRNA, a signal of about the same intensity was detected

Figure 3.3.1 Northern analysis of *bldG* and *orf3* transcripts. RNA (40 µg) was isolated from *S. coelicolor* plate cultures at various times post-inoculation (hours) as indicated. The RNA, along with the molecular weight marker (Marker III), was denatured with glyoxyl, separated on a 1.25% agarose gel, and transferred to Hybond™-N nylon membrane. The probe for *bldG* was a random primer labeled 207 bp PCR product internal to the *bldG* coding sequence, and a 428 bp random primer labeled PCR product was used to probe for *orf3*. Hybridizations using the *bldG* and *orf3*-specific probes were carried out at 65 °C in 50% formamide, and the same temperature was used for all washes. To control for RNA loading levels, the blot was hybridized with an end-labeled oligonucleotide probe (BKL54) specific for 16S rRNA. In this case, hybridization and washing were performed at 55 °C without formamide. Blots were exposed to a phosphorscreen overnight for visualizing *bldG* and *orf3* transcripts, and for four hours when visualizing the 16S rRNA. After scanning the screen in a PhosphorImager, the images were analyzed using Imagequant™ software.



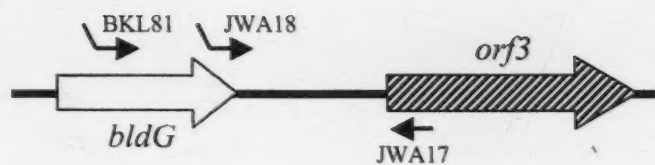
in all lanes, indicating that the variation in *bldG* and *orf3* transcript levels is not due to differences in RNA loading levels. Quantitative analysis of the two *bldG* transcripts indicated that the smaller transcript is present at about a 2-3 fold higher concentration than the larger transcript.

3.3.2 5' S1 nuclease mapping of *orf3* transcripts

S1 nuclease protection assays were carried out to confirm that the *orf3* gene is co-transcribed with *bldG* as opposed to being transcribed from its own promoter. The probe used was an end-labeled 182 bp PCR product (JWA17-18, see Table 2.5) which extended 121 bp upstream of the *orf3* start codon and contained a 10 nt non-homologous extension at one end to distinguish between full length probe protection and probe-probe re-annealing (see Figure 3.3.2). The RNA was hybridized for 3 hours at 63 °C with the probe, treated with S1 nuclease, and then analyzed by denaturing polyacrylamide gel electrophoresis along with a sequencing ladder generated using the same oligonucleotide (JWA17) used to synthesize the probe. The major S1 product observed at all time points examined was 172 nt in size, which is equivalent to the full length probe excluding the 10 nt non-homologous extension. When a 411 bp probe (JWA17-BKL81) which extended further upstream (350 bp) of the *orf3* start codon was used for S1 mapping, the same results were observed, confirming that the *orf3* gene is not transcribed from a promoter within the intergenic region or within the 3' end of the *bldG* coding region.

Figure 3.3.2 S1 mapping of the intergenic region between *bldG* and *orf3*. (A) The probes used included a 182 bp PCR product generated using JWA17 and JWA18 as primers, and a 411 bp PCR product generated using the primers JWA17 and BKL81. The location and binding direction of the primers is indicated by the black arrows, along with the 10 nt non-homologous extensions which are shown as curved lines extending from the arrows. RNA (40 µg) was isolated from surface-grown J1501 cultures at 24 and 36 hours post-inoculation, and was hybridized separately with the JWA17-18 (B) and the BKL81-JWA17 (C) probes for 3 hours at 63 °C or 58 °C, respectively. The resulting DNA/RNA duplexes were treated with S1 nuclease, and then separated on a 6% sequencing gel along with a sequencing ladder (indicated by A,C,G,T) generated using the same oligonucleotide (JWA17) used to synthesize the probes. Control lanes on the gel include probe alone (no RNA) that went through the S1 procedure (Probe+S1), and probe alone that did not go through the S1 procedure (Probe-S1). The locations of the full length probe and the product resulting from full length protection are indicated.

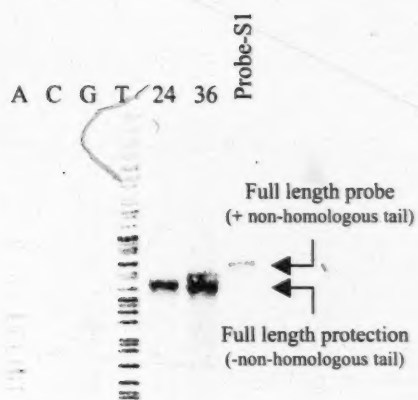
A



B



C



3.3.3 3' S1 nuclease mapping of *bldG* transcripts

To confirm the existence of the two *bldG* transcripts, S1 nuclease protection assays were carried out to map the location of the 3' end of the shorter transcript. Figure 3.3.3 shows how the probe used to map the 3' end was prepared. First, a 528 bp fragment was amplified by PCR using pAU66 as the template and the primers BKL62 and DBG17, and was cloned into pCR2.1TOPO to give pAU312. Digestion of pAU312 with *Hind*III and *Pvu*I resulted in release of an 801 bp fragment, and after purification, the fragment was digested with *Bgl*II and was incubated with Klenow enzyme, cold dNTPs (dATP, dGTP, dTTP) and α -³²P-dCTP in order to label the 3' end of the probe. The resulting ³²P-labeled probe was 705 bp in size, and consisted of 492 bp of *S. coelicolor* DNA and 213 bp of vector DNA, which served as a non-homologous extension to distinguish between probe-probe re-annealing and full length probe protection. The probe was hybridized with RNA for 3 hours at 61 °C, treated with S1 nuclease, and analyzed by electrophoresis on a 6% sequencing gel along with a sequencing ladder generated using BKL62 (the sequencing ladder was used only as a means of determining the size of the resulting S1 products, and does not indicate the nature of the base which corresponds to the 3' end of the transcript). The results (Figure 3.3.4) show two major S1 products, the larger of which corresponded to full length protection of the probe, and therefore confirmed the existence of a *bldG-orf3* polycistronic transcript. The smaller S1 product had a size of 162 bp, which corresponded to an mRNA 3' end that terminated immediately after the second of two inverted repeats found within the *bldG-orf3* intergenic region (see Figure 3.3.7). A 3' end located within this region would give transcript sizes of 507 nt (when initiated from P1, see Section 3.3.3) and 548 nt (when

Figure 3.3.3 Strategy for designing a probe to detect the 3' end of the *bldG* monocistronic transcript. A 528 bp fragment including the 3' end of the *bldG* and the 5' end of the *orf3* coding regions was amplified by PCR using the primers BKL62 and DBG17. The locations and binding directions of the primers are indicated as black arrows, with the BKL62 non-homologous extension indicated by the line extending from the arrow. The fragment was cloned into pCR2.1TOPO to give pAU312, and was then liberated as an 801 bp fragment by digestion with *Hind*III and *Pvu*I, which cut at vector-specific sites (indicated by ▼). Labeling of the probe at the 3' end (indicated by *) was accomplished by digesting the gel-purified 801 bp fragment with *Bgl*II and blunting with Klenow enzyme and α -³²P-dCTP. *S. coelicolor* DNA in the probe (492 bp) is represented by the hatched box, whereas vector-specific DNA (213 bp) which functioned as a non-homologous extension to differentiate between probe-probe re-annealing and full length probe protection is indicated by the thin black line.

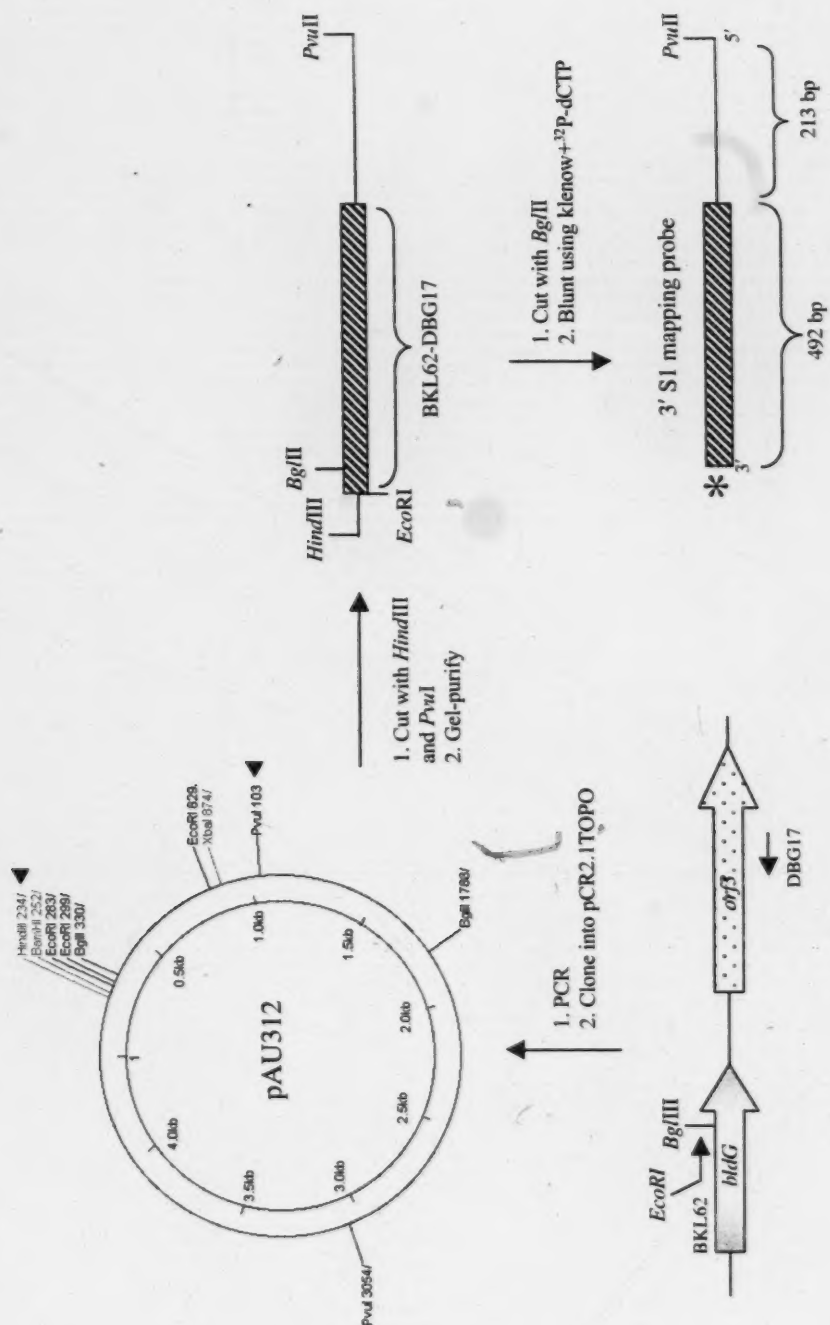
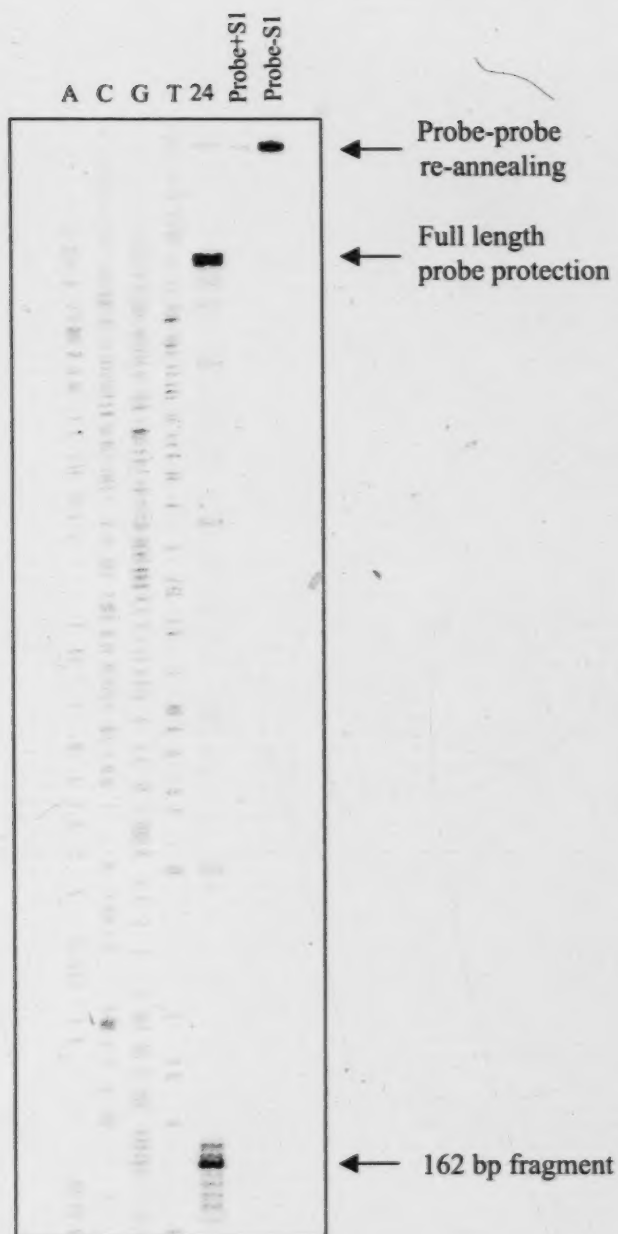


Figure 3.3.4 S1 nuclease mapping of the 3' end of the *bldG* monocistronic transcript. RNA (40 µg) isolated at 24 hours post-inoculation from surface-grown *S. cerevisiae* J1501 was hybridized with a 705 bp probe, and then treated with S1 nuclease. The products were separated on a 6% sequencing gel along with sequencing reactions (indicated as A,C,G,T) generated using BKL62 which anneals just upstream of the 3' end of the probe. Control lanes include probe alone that went through the S1 procedure (Probe+S1) and probe alone that did not go through the S1 procedure (Probe-S1). The positions of the full length probe, the product resulting from full length protection of the probe, and the 162 nt product used to map the 3' end of the *bldG* monocistronic transcript are indicated.

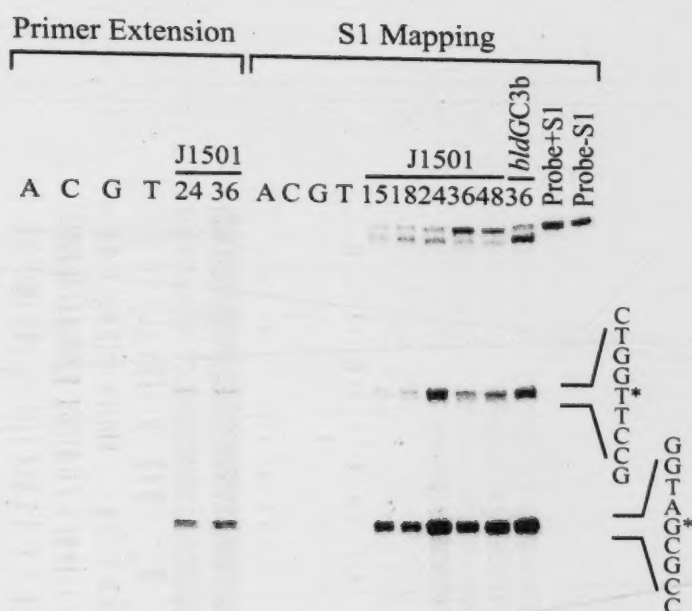


initiated from P2, see Section 3.3.3), which agrees with the size the smaller transcript as observed by northern analysis.

3.3.4 5' S1 nuclease mapping of *bldG* transcripts

High resolution S1 nuclease mapping of 5' ends was performed to map the location of the *bldG* transcription start site(s) as well as to confirm the timing of expression of *bldG* transcripts. RNA isolated at various times post-inoculation from J1501 was hybridized with a 264 bp PCR-generated probe (JWA20-DBG14) for 3 hours at 56 °C, and was then treated with S1 nuclease. The probe included a 10 nt non-homologous extension in order to differentiate between full length probe protection and probe-probe re-annealing. To control for RNA loading levels, the RNA samples were first subjected to northern analysis using the 16S rRNA probe, and aliquots showing equivalent signals were used for S1 nuclease mapping. The resulting S1 products were separated on a 6% polyacrylamide sequencing gel along with a sequencing ladder generated using the oligonucleotide (DBG14) used to generate the probe. The experiment was performed using RNA isolated from three independent time courses, and representative results are shown in Figure 3.3.5. Two major S1 nuclease products were observed with sizes of 152 nt and 193 nt and corresponding to transcription start sites located 82 and 123 nt upstream of the *bldG* start codon. A third S1 product was also observed which was slightly smaller than the full length probe, however this band most likely represents an artifact of the S1 procedure, since the same band was detected in the control lane (Probe+S1) of repeated S1 mapping experiments using the same probe (not shown). The start site which was proximal to the translation start codon (designated P1)

Figure 3.3.5 Detection of 5' ends of *bldG* mRNA by primer extension analysis and high resolution S1 nuclease mapping. Total RNA (40 µg) was isolated from surface-grown *S. coelicolor* J1501 and *bldGC3b* (see Section 3.3.4) at various time points (hours post-inoculation) as indicated. For primer extension analysis, the end-labeled oligonucleotide DBG15 (anneals just downstream of the *bldG* start codon) was hybridized with the RNA, and was then extended using AMV reverse transcriptase (Roche). The resulting cDNA was separated on a 6% sequencing gel along with sequencing reactions (labeled A,C,G,T) generated using DBG15 as the primer. For S1 nuclease mapping, the RNA was hybridized with an end-labeled 264 bp probe (JWA20-DBG14), treated with S1 nuclease, and then separated on a 6% sequencing gel along with sequencing reactions (A,C,G,T) generated using DBG14. Control lanes shown include probe alone that went through the S1 procedure (Probe+S1) and probe alone that did not go through the S1 procedure (Probe-S1). (*) denotes the most probable transcription start sites.



was located just downstream of a putative -10 sequence similar to those found in *E. coli*-like *Streptomyces* promoters (Strohl, 1992). A putative -35 sequence was also present, which was separated from the -10 sequence by a spacing of 16 nt (see Figure 3.3.7). For the more distal transcription start site (designated P2), a putative -10 sequence was also observed, however an appropriately spaced -35 sequence was not found (Figure 3.3.7).

A comparison of the product intensities over the time course confirmed the northern data in showing that the promoters are expressed at lower levels during vegetative growth, and are upregulated when aerial hyphae and pigmented antibiotics are visible. The relative intensities of the two products at each time point correlated well with the relative intensities of the short and long *bldG* transcripts observed by northern analysis, suggesting that initiation of transcription from the more distal promoter may give rise to the larger transcript while transcription initiation from the more proximal promoter leads to synthesis of the shorter transcript. Alternatively, both promoters may give rise to both transcripts, and transcription from either promoter may differentially increase or decrease under certain conditions, such as in response to some unknown signal or to stress experienced by the organism.

It has previously been shown that the *spoIIA* operon of *B. subtilis* is transcribed from two separate promoters, one of which is controlled by σ^F itself (Schuch & Piggot, 1994). Inactivation of the SpoIIAA anti-anti-sigma factor leaves σ^F in an inactive complex with SpoIIAB, and therefore transcription from this σ^F -dependent promoter does not occur. To determine whether one or both of the *bldG* promoters is likewise dependent on BldG, S1 mapping was performed using RNA isolated from the *bldGC3b* null mutant strain previously constructed by inserting the thiostrepton resistance gene

(*tsr*) into a *Bgl*III site in the *bldG* coding region (see Table 2.2). As shown in Figure 3.3.5, the same mRNA 5' ends were detected with the same relative abundance in the mutant strain, suggesting that loss of BldG activity does not affect transcription from either *bldG* promoter.

3.3.5 Primer extension analysis of *bldG* transcripts

To confirm that *bldG* is transcribed from two separate promoters, primer extension analysis was carried out using an end-labeled oligonucleotide primer (DBG15, see Table 2.5) that was designed to hybridize immediately downstream of the *bldG* start codon. RNA (40 μ g) was hybridized to the primer for 1 hour at 55 °C, followed by primer extension using AMV reverse transcriptase. The resulting cDNA products were subsequently separated on a 6% sequencing gel along with a sequencing ladder generated using DBG15. Two cDNA products were observed with sizes of 102 nt and 143 nt (Figure 3.3.5), and which corresponded to the same transcription start sites determined using S1 nuclease mapping. These results therefore verify that the S1 products observed were in-fact due to the presence of two transcription start sites as opposed to being artifacts of the S1 procedure.

3.3.6 Effect of salt shock on *bldG* expression

It has previously been shown in *S. coelicolor* that transcription of the *sigH* and *sigB* genes, encoding sigma factors which resemble the stress-response sigma factor σ^B of *B. subtilis*, are induced in response to salt shock. In both cases, the promoters that are induced by salt shock are transcribed by RNA polymerase containing the corresponding

sigma factor itself (Cho *et al.*, 2001; Sevcikova *et al.*, 2001). Alignment of *sigBP1* and *sigHP2* promoter sequences with that of *ssgB* [which is transcribed by SigH; (Kormanec & Sevcikova, 2002)] and *bldGP1* showed a significant degree of similarity between all of the promoter sequences (Figure 3.3.6A), suggesting that the *bldGP1* promoter may also be induced in response to salt shock. To investigate this, J1501 cultures were grown in liquid NMMP medium containing mannitol as the carbon source, and were then induced with 2.5% NaCl (final concentration), and incubated for 60 and 90 minutes before harvesting the mycelia for RNA isolation. Northern analysis was then performed using the *bldG*-specific probe BKL63-64 (see Section 3.3.1) in order to determine whether *bldG* transcript levels increased in response to salt induction. As shown in Figure 3.3.6B transcript levels did not change significantly after induction with NaCl, suggesting that *bldG* expression is not induced by salt stress.

In summary, the transcriptional data collected from S1 nuclease mapping experiments, primer extension analysis, and northern analysis, indicate that *bldG* is expressed from two promoters located 82 and 123 nt upstream of the start codon, and that it is expressed both as monocistronic and a polycistronic transcript, with the latter transcript including the downstream *orf3* gene (Figure 3.3.7). Analysis of the intergenic region between *bldG* and *orf3* revealed two inverted repeats that could give rise to stem-loop structures with ΔG values of -22.2 and -31.3 kcal (-93 and -131 kJ), respectively, either of which could play a role in mRNA stability, mRNA processing or in rho-independent transcription termination.

Figure 3.3.6 (A) Comparison of the *bldGP1* promoter with the *sigBP1*, *ssgB*, and the *sigHP2* promoters of *S. coelicolor*. The -10 and -35 sequences are indicated, as well as the transcription initiation sites (+1) and the sizes of the spacer regions (n) between the initiation site and the -10 sequence, and between the -10 and -35 sequences.

Nucleotides comprising the -10 and -35 sequences that are conserved in all promoters are indicated in red, while those that are conserved in three of the four promoters are indicated in green. (B) Analysis of *bldG* transcript levels in response to salt shock. RNA (40 µg) was isolated from *S. coelicolor* J1501 liquid cultures grown in NMMP medium before (0 minutes) and after (60 and 90 minutes) the addition of 2.5% NaCl (final concentration). *bldG* transcript levels were then analyzed by northern analysis using a ³²P-random primer labeled internal *bldG* fragment (BKL63-64, see Section 3.3.1). To control for RNA loading levels, the same blot was probed with the end-labeled BKL54 oligonucleotide probe specific for 16S rRNA.

(A)

	<u>-35</u>		<u>-10</u>		<u>+1</u>
<i>sigBP1</i>	G G G	n = 13	G GTAG	n = 6	GG
<i>bldGP1</i>	G GCA	n = 16	TTGAAT	n = 5	CG
<i>ssgB</i>	G T A	n = 14	T GCAT	n = 7	TC
<i>sigHP2</i>	GGT	n = 16	G GTAC	n = 8	GG

(B)

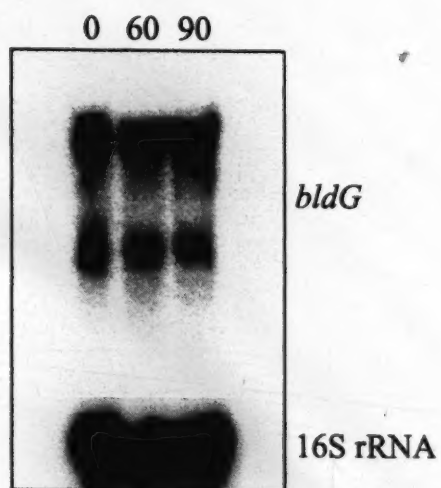


Figure 3.3.7 Compilation of transcriptional features of the *bldG* locus. The deduced amino acid sequences of *bldG* and the partial amino acid sequences of SCH5.13 and *orf3* are indicated below the nucleotide sequence. The proposed transcription start sites (P1, P2; ○→), and the -10 and -35 sequences are shown, as well as the putative ribosome binding sites (RBS, indicated in bold) and the start codons for both *bldG* and *orf3* (indicated by the clear boxes). The two inverted repeats downstream of the *bldG* stop codon (*) are indicated by the arrows, and the proposed location of the 3' end of the *bldG* monocistronic transcript is indicated by the gray box. Nucleotide positions on the right are indicated relative to the first nucleotide of the *bldG* start codon (designated +1).

GCCGGTCCAGGAGCATGCCCGGTTCCGGCCGGGAGCCGGGCTCCGCCG -195
 R D L L fm SCH5.13
 ←
 GGGATCGATCGGGTCGGTGATTCTTGGCCATCGGCACCGAGTCTGTCA -147
 -10 P2 -35
 CTGGCGTGACGGACAATGGGACCAAGGCGTCGTGCACGCCCTGCCGGTA -99
 -10 P1
 AGTGATTGAATGCCATCGCGGCTGGCGAACCGTCCTGGGGGCTTCAAG -51
 CCGAGGTGTCCCGAGGGACGACCGCTCGATAGCAAGGTGCTGGAGGAT -3
 CCTGACCTGTCCCTGTGACCCGTACCGTCGGCGATCGTACGGTC +45
 fm D L S L S T R T V G D R T V
 CTCGAGGTCGGTGGCGAAATTGACGTATACACCGCGCCCAAGCTGCGT +93
 V E V G G E I D V Y T A P K L R
 GAGCAGCTGGTCGAGCTCGTGAACGACGGGAGTTTCCACCTCGTCGTC +141
 E Q L V E L V N D G S F H L V V
 GACATGGAGGGCGTGGACTTCCTCGACTCCACAGGGCTCGGCGTGCTG +189
 D M E G V D F L D S T G L G V L
 GTGGGTGGACTGAAGCGGGTGCGTGCCCATGAGGGCTCGCTGCGGCTG +237
 V G G L K R V R A H E G S L R L
 GTCTGCAACCAGGAGCGCATTCTCAAGATCTTCCGTATCACCGGCCTC +285
 V C N Q E R I L K I F R I T G L
 ACCAAGGTGTTCCCCATTACACCTCGGTGAGGAAGCGGTGGCGGCC +333
 T K V F P I H T S V E E A V A A
 ACCGACTGACGACCGGTTCCGGGCCTCTCGGGGGCCCGGGACGCAAGTA +381
 T D *
 GAACAAGGGGGGTCCGGGCTGTGCGGCAGCCCGGACCCTCGAAAGCAGC +429
 CCCGCAGTTCCGAGGGGATGATGGCCACCGTCGAA +466
 orf3 fm A T V E

3.4 Protein Overexpression and Purification

Overexpression and purification experiments were next performed in order to obtain large quantities of BldG and ORF3 proteins for use in biochemical studies and for raising polyclonal antibodies in rabbits. BldG was overexpressed as a MBP-fusion protein, whereas ORF3 was overexpressed as a 10× histidine-tagged protein, and the antibodies raised against the two purified fusion proteins were used to examine the relative levels of BldG and ORF3 proteins in *S. coelicolor* crude extracts during development. The BldGS57A mutant protein was also expressed as a MBP-fusion protein in order to examine the effects of the mutation using biochemical studies.

3.4.1 Overexpression and purification of MBP-BldG and MBP-BldGS57A

To overexpress the BldG protein, the *bldG* coding region was cloned into the pMAL-c2X vector downstream of the *male* gene (encoding MBP). The cloning strategy was designed such that the MBP-tag could be removed from the purified fusion protein by cleavage with Factor Xa, leaving no extra residues on the N-terminus of BldG. Briefly, a 351 bp *bldG*-containing fragment was first amplified by PCR using pAU64 as the template and the primers DBG20 (containing a single non-homologous residue to change the BldG start codon from GTG to ATG) and DBG5 (see Table 2.5). After purification, the resulting product was incubated with T4 DNA polymerase in order to remove any 3' non-homologous A residues added on by Taq polymerase, then digested with *HindIII*, and ligated into the pMAL-c2X vector that had been digested with *XmnI* and *HindIII*. Ampicillin-resistant *E. coli* DH5α transformants that arose were then screened for the desired clone according to the pMAL Expression and Purification

manual (New England Biolabs), and a single clone, designated pAU308, was sequenced to ensure no PCR-induced mutations were present within the insert.

Expression of the MBP-BldG fusion protein was carried out in *E. coli* DH5 α , and was induced from the *ptac* promoter by the addition of 0.3 mM IPTG (final concentration). Cells were incubated at room temperature for 6 hours after induction, since it was found that incubating at 37 °C reduced the amount of soluble protein obtained. After incubation, the cells were pelleted, frozen, then thawed, and sonicated in order to lyse the cells. Uninduced and IPTG-induced total extracts were analyzed by SDS-PAGE, and a protein band of about 55 kDA was observed in the induced extracts, which is the expected molecular weight of the desired fusion protein (Figure 3.4.1). Analysis of soluble and insoluble fractions revealed that ~30% of the fusion protein was present in soluble form, and this soluble protein was purified to homogeneity by affinity chromatography using an amylose column, followed by FPLC using a strong anion exchange column (Figure 3.4.2).

To express the BldGS57A mutant protein as a MBP-fusion protein, PCR was first performed using pAU320 as the template and DBG4 and DBG5 as primers (see Table 2.5). The resulting 357 bp product was digested with *Bam*HI and *Hind*III and ligated into the *Bam*HI and *Hind*III sites of pMAL-c2X to give pAU321. Overexpression and purification of the fusion protein was then carried out as described for the wild type MBP-BldG fusion protein, except that the purified protein was not subjected to FPLC following affinity chromatography (see Figure 3.4.3).

Figure 3.4.1 Overexpression of MBP-BldG. MBP-BldG was overexpressed in *E. coli* DH5 α from the *ptac* promoter induced with 0.3 mM IPTG. Total protein extract before the addition of IPTG (uninduced) was prepared by pelleting 1 ml of culture and resuspending in 50 μ l SDS-PAGE sample buffer, while total extract after IPTG addition (induced) was prepared by pelleting 0.5 ml of culture and resuspending the cells in 100 μ l sample buffer. Aliquots (10 μ l) were then analyzed on a 10% SDS-PAGE stained with Coomassie Blue. The remaining induced cells were pelleted and lysed by sonication, and soluble and insoluble fractions were separated by centrifugation. Aliquots of each fraction (10 μ l) were mixed with sample buffer and were separated in a 10% SDS-PAGE and visualized by staining with Coomassie Blue. SDS-PAGE broad range standards were used as the molecular weight marker, the band sizes of which are indicated, along with the position of MBP-BldG.

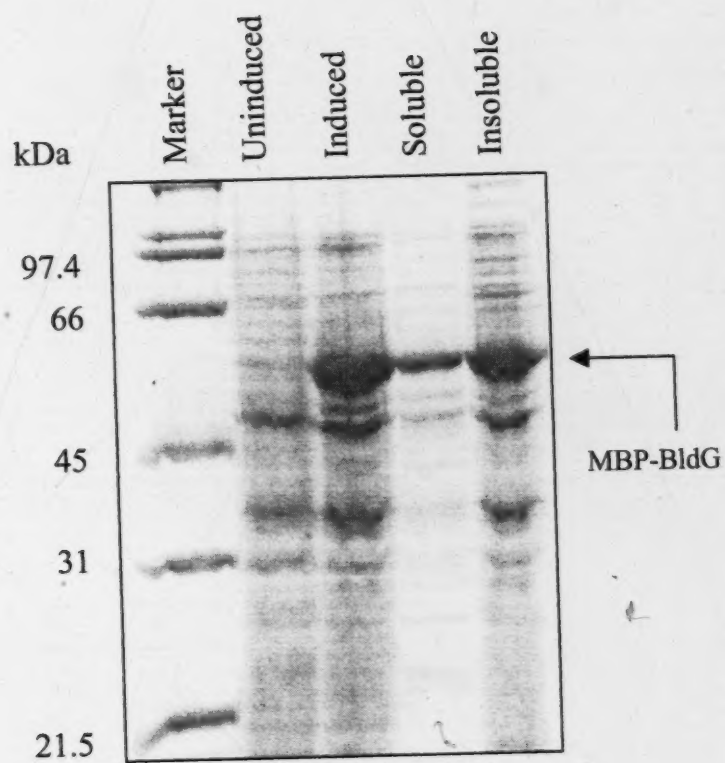


Figure 3.4.2 Purification of MBP-BldG. Soluble *E. coli* extract containing MBP-BldG was diluted 1 in 4 in column buffer and loaded onto an amylose affinity column. After washing the column with 10 volumes of column buffer, the fusion protein was eluted with 10 mM maltose. Fractions collected (1 ml) were analyzed by separating 10 μ l on a 10% SDS-PAGE and staining with Coomassie Blue, and those containing the fusion protein were then combined and loaded onto an FPLC strong anion exchange column. The fusion protein was eluted from the FPLC column using a gradient of 25-500 mM NaCl, and 5 μ l of each fraction collected (indicated as 1-5) was analyzed by SDS-PAGE. SDS-PAGE broad range standards were used as the molecular weight marker, the band sizes of which are indicated in kDa.

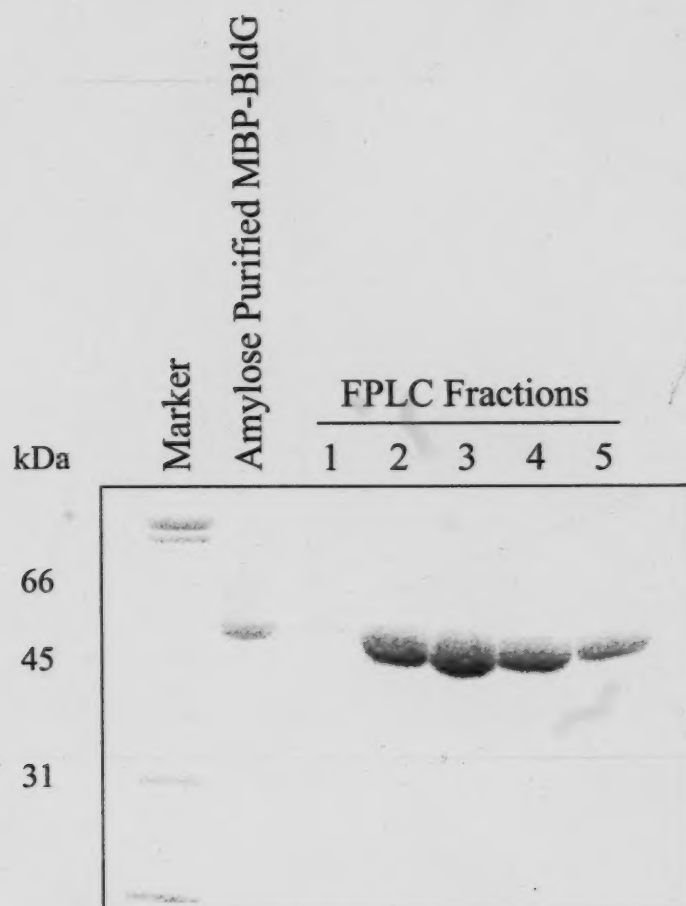
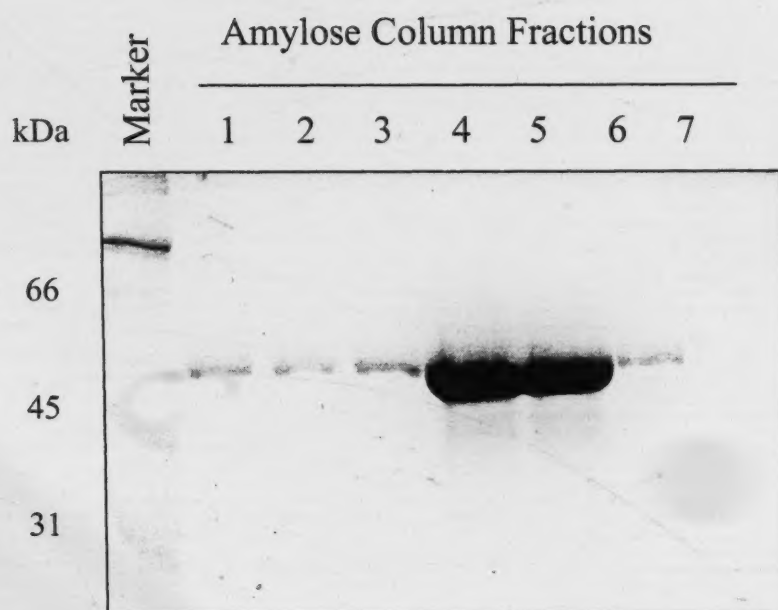


Figure 3.4.3 Purification of MBP-BldGS57A. MBP-BldGS57A was overexpressed in *E. coli* DH5 α after the addition of 0.3 mM IPTG. Soluble extract was diluted with column buffer and loaded onto an amylose column, after which the column was washed with 10 volumes of column buffer. The fusion protein was then eluted from the column by the addition of 10 mM maltose in column buffer, and fractions collected (1 mL each; indicated as 1-7) were analyzed by separating 10 μ l on a 10% SDS-PAGE, and then staining the gel with Coomassie Blue. SDS-PAGE broad range standards were used as the molecular weight marker, the band sizes of which are indicated.



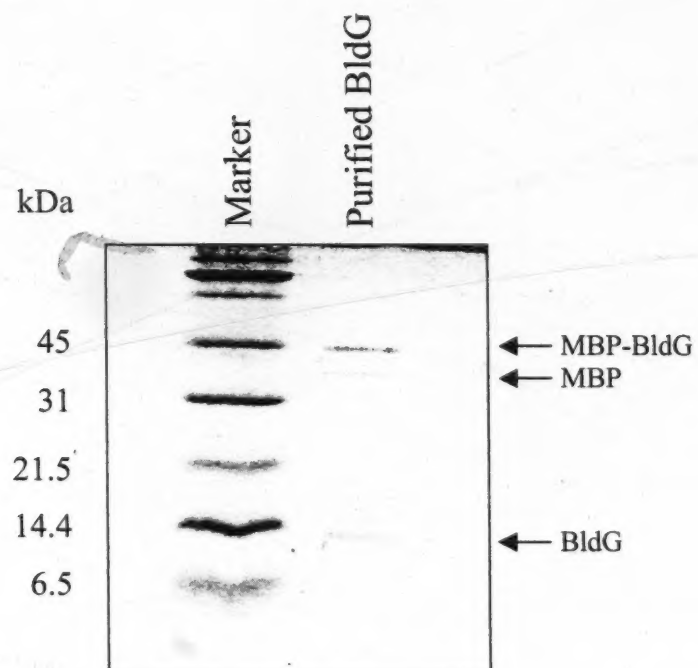
3.4.2 Purification of BldG

Purified BldG protein was obtained by cleaving the MBP-tag off of the MBP-BldG fusion protein using Factor Xa. The reaction was carried out in the presence of 0.05% SDS which increased the amount of untagged BldG protein obtained, and the BldG protein was then purified by separating the reaction mixture on a Superdex-75 FPLC size-exclusion column. Analysis of the fractions eluted from the column revealed that the BldG-containing fractions also contained some uncleaved MBP-BldG as well as some MBP protein. To further purify the untagged BldG protein, the fractions were combined and incubated overnight with amylose resin, after which the supernatant was removed, and an aliquot was analyzed by SDS-PAGE. As shown in Figure 3.4.4, incubation with the amylose resin did not completely remove all of the uncleaved MBP-BldG or the MBP, and it was estimated that about 1/3 of the protein present in the supernatant was untagged BldG. Since the untagged BldG protein was found to be inactive in *in vitro* phosphorylation assays (see later), it was used only for generating a standard curve for measuring BldG protein levels in *S.coelicolor* crude extracts by western immunoblotting (see Section 3.5.1).

3.4.3 Overexpression and purification of His₁₀-ORF3

Purified ORF3 protein was obtained by cloning the *orf3* coding region into a modified pET30a(+) vector downstream of a 10 × histidine-tag (see Table 2.3). Briefly, a 556 bp fragment containing the *orf3* coding region was released from pAU317 (Table 2.4) by digestion with *Nde*I and *Eco*RI, and after purification, this fragment was ligated

Figure 3.4.4 Purification of BldG. MBP-BldG (~7.5 mg) was incubated overnight at room temperature with Factor Xa in the presence of 0.05% SDS, after which the reaction mixture was concentrated and was loaded onto a Superdex-75 size exclusion FPLC column. The fractions eluted from the column were analyzed by SDS-PAGE, and those fractions containing untagged BldG protein were combined and were incubated with amylose resin in order to remove any uncleaved MBP-BldG or any free MBP tag that co-purified with BldG. The resin was then pelleted and an aliquot of the supernatant (10 μ) was analyzed by SDS-PAGE on a 15% polyacrylamide gel. SDS broad range standards (Bio-Rad) was used as the molecular weight marker, the band sizes of which are indicated in kDa, as well as the position of untagged BldG, free MBP tag, and uncleaved MBP-BldG.



into the modified pET30a(+) vector to give pAU318. The presence of the insert was then verified by restriction digestion and DNA sequence analysis.

Expression of the fusion protein was carried out in *E. coli* BL21(DE3)pLysS under control of the IPTG-inducible T7 promoter. Cultures were grown and induced with IPTG (0.5 mM final concentration), and were then incubated for 2 hours at 37 °C. Figure 3.4.5 shows that under the conditions used, the amount of fusion protein expressed was very small, failing to yield a visibly induced band when total lysates were analyzed by SDS-PAGE. Purification of His₁₀-ORF3 was by Ni-chelate affinity chromatography under native conditions, where protein extracts were prepared in a native buffer and were incubated with Ni-NTA resin, followed by elution of the fusion protein from the resin using high levels of imidazole (Figure 3.4.5). This protein preparation was subsequently used in biochemical studies where the presence of native ORF3 protein was required. For the preparation of ORF3-specific antibodies, on the other hand, His₁₀-ORF3 was purified under denaturing conditions in the presence of 8M urea (Figure 3.4.6), since native protein was not necessary in this case, and higher levels of fusion protein could be obtained under these conditions than when native conditions were used. The molecular weight of the fusion protein was estimated by SDS-PAGE to be about 30 kDa, which is significantly larger than that predicted from the amino acid composition (14.9 kDa). This is most likely due in part to the presence of the 10 × histidine tag, which is known to slow the migration of proteins through SDS-polyacrylamide gels (Qiagen manual). Also, the overall charge and/or shape of the protein may be affecting the movement of the protein through the gel. Purification under denaturing conditions routinely resulted in co-purification of a larger molecular weight protein (Figure 3.4.6), however since the desired

Figure 3.4.5 Overexpression and purification of native His₁₀-ORF3. Overexpression of His₁₀-ORF3 was carried out in *E.coli* BL21(DE3)pLysS from the T7 promoter after induction with 0.5 mM IPTG. Total cell extracts before IPTG addition (uninduced) were prepared by pelleting 1 ml of culture and resuspending the cells in 50 µl SDS-PAGE sample buffer, while cell extracts after IPTG addition (induced) were prepared by pelleting 0.5 ml of culture and resuspending the cells in 100 µl sample buffer. Aliquots (12 µl) were then analyzed on a 12% SDS-PAGE. Batch purification of native soluble His₁₀-ORF3 was performed by mixing soluble extract with Ni-chelate affinity resin, and then loading the resin mixture into an empty column. After washing the column with 8 volume of native wash buffer, the fusion protein was eluted with elution buffer containing 250 mM imidazole, and fractions collected (10 × 0.5 ml) were analyzed by SDS-PAGE. Those containing the desired protein were pooled and concentrated, and a 5 µl aliquot was separated on a 12% SDS-PAGE to check the purity of the protein.

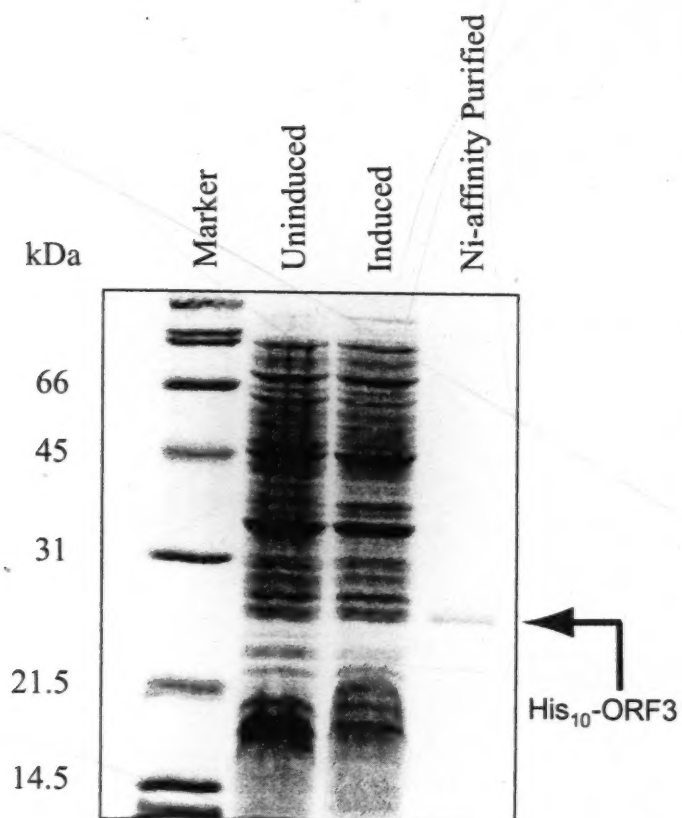
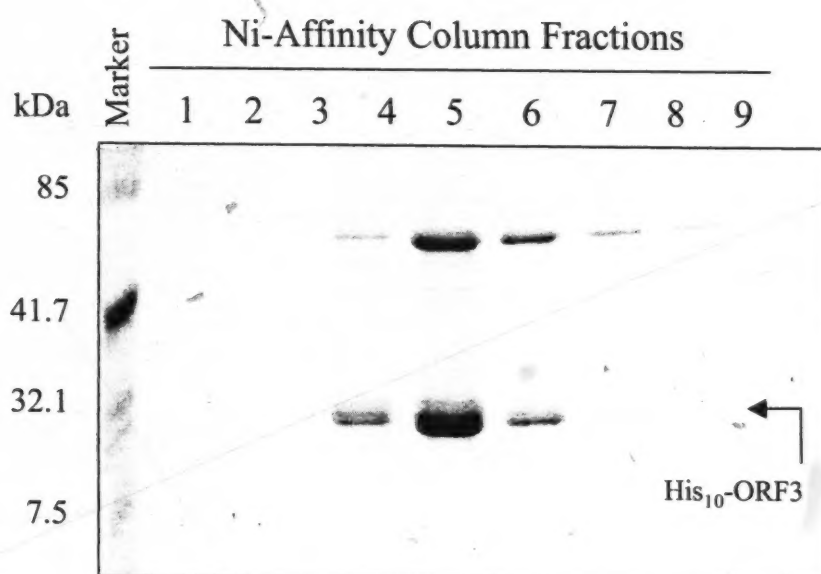


Figure 3.4.6 Purification of denatured His₁₀-ORF3. Total *E. coli* cell extract prepared under denaturing conditions and containing His₁₀-ORF3 protein was incubated with Ni-chelate affinity resin, and the mixture was loaded into an empty column. After washing the resin with 10 column volumes of denaturing wash buffer, the fusion protein was eluted with denaturing elution buffer, and 10 µl aliquots of the fractions collected (1 ml each) were analyzed by SDS-PAGE (indicated as 1-9). Those fractions containing the desired protein were then pooled and concentrated. Kaleidoscope marker was used as the molecular weight marker, and the marker band sizes are indicated, as well as the position of the His₁₀-ORF3 band.



protein was to be excised from the gel in preparation for injection into rabbits for antibody generation, the presence of this second protein was not a concern.

3.5 Protein Analysis

3.5.1 Western analysis of BldG and ORF3

Transcriptional analysis of the *bldG* locus had indicated that both *bldG* and *orf3* are expressed at all time points examined, with elevated levels observed starting at 24 hours post-inoculation when both aerial hyphae and antibiotics are visible. Moreover, *bldG* monocistronic and *bldG-orf3* polycistronic transcripts were detected whereas no *orf3* monocistronic transcripts were observed, suggesting an excess of BldG over ORF3 protein during development. To determine whether this expression pattern observed could also be seen at the level of protein production, western analysis was performed to examine the levels of BldG and ORF3 proteins at various stages of development. *S. coelicolor* J1501 cell extracts isolated over a 48 hour period were separated on a 12 or 15% SDS-PAGE, and were then transferred to PVDF membrane. Purified BldG protein and extract isolated from the $\Delta bldG$ 1DB mutant strain at 36 hours post-inoculation were separated along with the J1501 extracts to serve as positive and negative controls, respectively, for BldG detection. In the case of ORF3, the positive control was soluble *S. coelicolor* extract isolated from a strain in which ORF3 was overexpressed (see Section 2.6.8). The membranes were incubated with either anti-MBP-BldG antibody at a dilution of 1 in 20 000, or with anti-His₁₀-ORF3 antibody at a dilution of 1 in 5 000. For quantification of the resulting BldG and ORF3 bands, known amounts of purified BldG and His₁₀-ORF3 were included on the gels, and were used to generate a standard curve

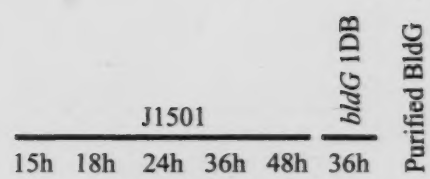
for each protein based on the measured band intensities. The results, shown in Figure 3.5.1A, indicate that the BldG protein could be detected in J1501 extracts at all time points examined, with the highest levels being observed starting at around 24 hours, which correlates with the levels of *bldG* transcripts. On the other hand, no BldG protein was detected in extract isolated from $\Delta bldG$ 1DB, which is consistent with this mutant having a deletion of 84 of the 113 amino acids from BldG. The ORF3 protein band was again observed to have a molecular weight larger than expected (~25-30 kDa), which suggests that the protein shape and/or charge does in-fact cause it to move slower than expected through polyacrylamide gels. The protein levels were also found to increase starting at 24 hours, however levels began to drop again by 36 hours, and very little ORF3 protein was detected at 48 hours, when transcript levels were still found to be quite high (Figure 3.5.1B). The absence of ORF3 protein in the 48 hour sample was not due to loss of the sample during loading of the gel or transfer to membrane, since a cross-reacting band could be detected in the same lane with the same intensity as in other lanes (Figure 3.5.1C). Quantification of the BldG and ORF3 bands in each lane indicated that BldG is present in excess of ORF3 at all time points examined, with 13-45 fold differences in levels being observed. It was also demonstrated that the ORF3 protein was present in the $\Delta bldG$ 1DB 36 hour extract, indicating that the expression of ORF3 was not affected by the deletion of *bldG* in the mutant strain.

3.5.2 Western analysis of BldG in *bldG* mutant extracts

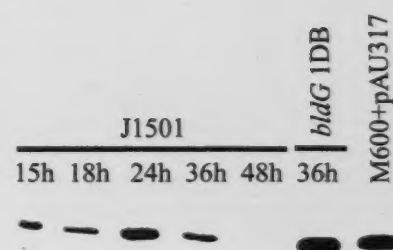
The point mutations identified in the *bldG* mutant strains resulted in production of a truncated 87 amino acid protein in the case of C103 and C536, and of a truncated 73

Figure 3.5.1 Western analysis of (A) BldG and (B) ORF3 proteins. Crude extracts (containing 40 µg protein) were isolated from *S. coelicolor* J1501 surface cultures at various times (hours post-inoculation) as indicated and were separated by SDS-PAGE. After transferring to PVDF membrane, the proteins were probed with either anti-MBP-BldG polyclonal antibodies at a dilution of 1 in 20 000, or with anti-His₁₀-ORF3 polyclonal antibodies at a dilution of 1 in 5 000, followed by Horseradish Peroxidase-conjugated secondary antibody at a dilution of 1 in 10 000. Detection of bands was by incubating the membrane in Enhanced Plus chemiluminescence reagent, followed by exposure to film for 1-60 minutes. Purified BldG protein and crude extract isolated from *S. coelicolor* Δ bldG 1DB were included as positive and negative controls, respectively, for BldG detection, while *S. coelicolor* extract containing overexpressed ORF3 (*S. coelicolor* M600 + pAU317) served as a positive control for ORF3 detection. A cross-reacting band detected with the anti-His₁₀-ORF3 antibodies (C) was used as a control for protein loading levels for the ORF3 western. Quantification of BldG and ORF3 protein bands was performed by generating a standard curve for each protein by measuring the band intensities of known amounts of purified BldG protein (0.068, 0.135, 0.271 µg) or His₁₀-ORF3 (0.0041, 0.0082, 0.0204 µg) which were included on the gels (not shown). Band intensities were measured using NIH Image software version 1.63.

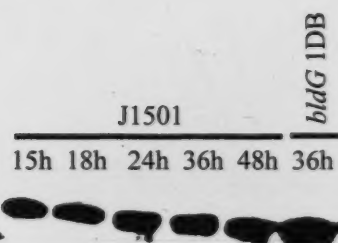
(A)



(B)



(C)



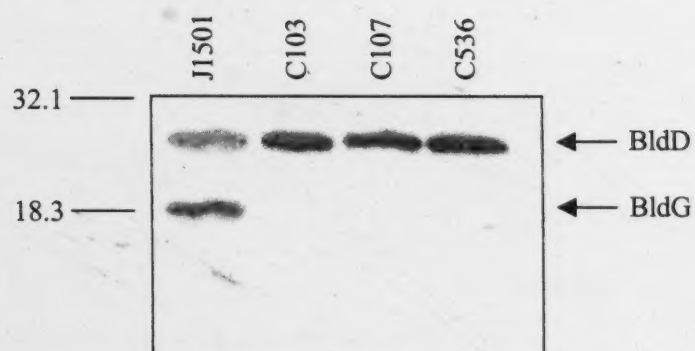
amino acid protein with an altered 18 amino acid sequence at the C-terminus in the case of C107 (see Section 3.2.2). To determine whether these mutations affected the stability of the BldG protein, western analysis was performed using extracts isolated from the C103, C107 and C536 mutant strains. As a control for protein loading levels, the extracts were simultaneously probed with antibodies against the BldD protein. Figure 3.5.2A shows that BldG could be detected in the wild type J1501 extract, but was not in any of the *bldG* mutant extracts. On the other hand, a band matching the expected molecular weight of BldD was observed in all lanes with similar intensities, confirming that the absence of BldG in the mutant lanes was not simply due to loss of the protein samples during loading of the gel or during transfer to membrane. These results, therefore, suggest that the point mutations resulted in a misfolded or non-functional BldG protein that was subsequently targeted for proteolysis.

3.5.3 Western analysis of ORF3 in *orf3* mutant extracts

To verify that the inability of the *orf3* mutants to be complemented by the *orf3* gene in pAU324 (see Section 3.2.7) was not due to a lack of *orf3* expression, western analysis was performed using 48 hour extracts isolated from the nine mutant strains grown on R2YE plates. The extracts were separated on a 12% SDS-PAGE, transferred to PVDF membrane, and probed with anti-His₁₀-ORF3 antibodies at a dilution of 1 in 5 000. Figure 3.5.3 shows that the ORF3 protein band could not be detected in extract from the mutant strains containing the plasmid vector alone (pSET Ω), however production of ORF3 protein was observed in extract from seven of the nine mutants that contained pAU324, confirming that the *orf3* gene in pAU324 is being expressed in these strains.

Figure 3.5.2 Western analysis of BldG in mutant cell extracts. Crude extracts (20 μ g) isolated from 48 hour *S. coelicolor* J1501, C103, C107 and C536 plate cultures (A) or from 24 and 36 hour plate cultures of Δ *bldG* 1DB containing pSET152, pAU69 (pSET152 containing *bldG*) or pAU325 (pSET152 containing *bldGS57A*) (B) were separated on a 15% SDS-polyacrylamide gel and were transferred to PVDF membrane. The membrane was then probed at the same time with anti-MBP-BldG antibodies and anti-His₆-BldD antibodies (1 in 20 000 dilution of each), the latter of which was used to control for protein loading levels. This was followed by incubation with horseradish peroxidase-conjugated secondary antibody at a dilution of 1 in 10 000, and visualization of the bands was by incubating the membrane in chemiluminescence reagent and exposing the membrane to film. Kaleidoscope pre-stained standards was used as the molecular weight marker, the band sizes of which are indicated, as well as the positions of BldG and BldD.

(A)



(B)

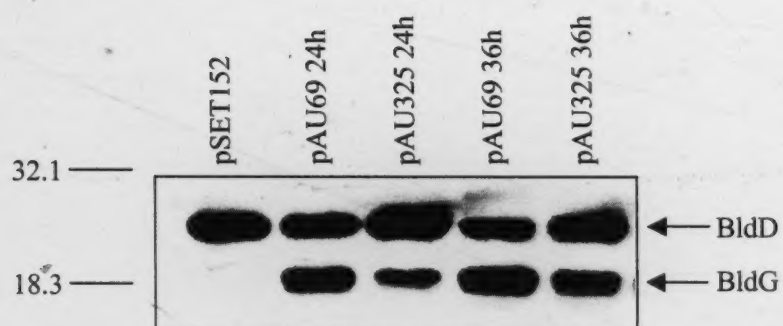
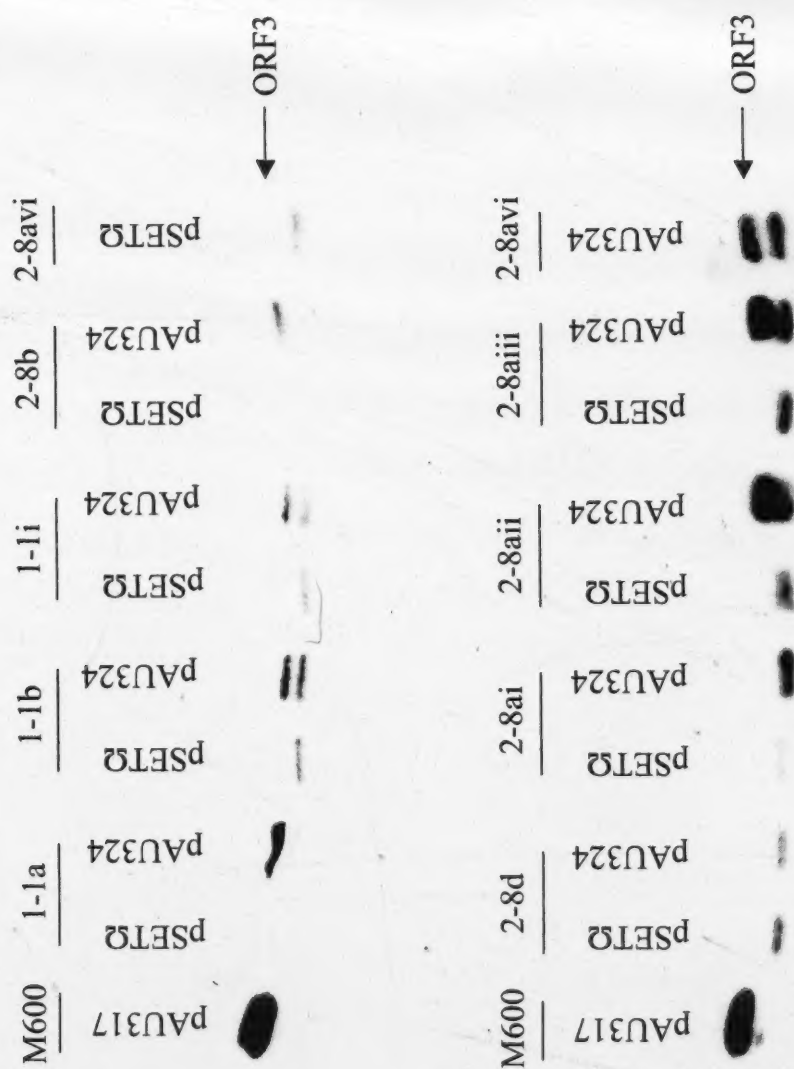


Figure 3.5.3 Western analysis of ORF3 in $\Delta orf3$ mutant extracts. Crude extracts were isolated from 48 hour plate cultures of the nine *orf3* deletion mutant strains containing either pSET Ω (control plasmid) or pAU324 (pSET Ω + *orf3*). Extracts (40 μ g total protein) were separated on a 12% SDS-polyacrylamide gel along with extract containing overexpressed ORF3 (M600 + pAU317; included as a positive control for ORF3 detection), and after transfer to PVDF membrane, the proteins were probed with anti-His₁₀-ORF3 antibodies at a dilution of 1 in 5 000, and then with horseradish peroxidase-conjugated secondary antibody at a dilution of 1 in 10 000. Visualization of the bands was by incubating the membrane with chemiluminescence reagent, and then exposing the membrane to film for 5 minutes. The position of the ORF3 protein is indicated.



On the other hand, production of ORF3 protein could not be detected in two of the nine mutants (2-8d and 2-8ai) that were complemented with pAU324.

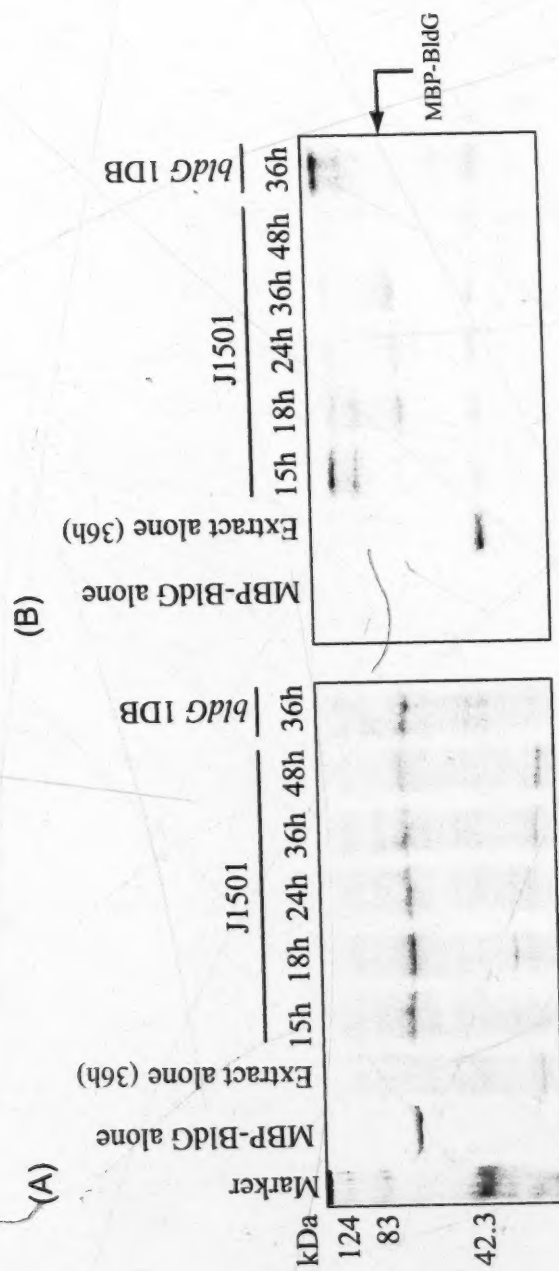
3.6 Phosphorylation Studies

The BldG homologs SpoIIAA and RsbV of *Bacillus subtilis* are known to be regulated by phosphorylation, since the phosphorylated forms of these proteins are unable to bind to their cognate anti-sigma factors, and are therefore unable to reverse the anti-sigma factor-mediated inhibition of the target sigma factor (Alper *et al.*, 1996; Dufour & Haldenwang, 1994; Duncan *et al.*, 1996; Magnin *et al.*, 1996). Since the known phosphorylation site in these proteins is conserved in BldG, it seemed possible that BldG might also be post-translationally-regulated by phosphorylation in *S. coelicolor*. Studies were therefore undertaken to explore whether BldG could be phosphorylated *in vitro* by *S. coelicolor* crude extracts, and whether phosphorylated BldG could be detected *in vivo*. As well, the effect of the S→A mutation at position 57 on the phosphorylation of BldG was investigated.

3.6.1 *In vitro* phosphorylation assays

In vitro phosphorylation reactions were performed to determine whether BldG could be phosphorylated by a component of *S. coelicolor* crude extract. Purified MBP-BldG was incubated with γ -³²P-ATP and *S. coelicolor* crude extracts isolated at various stages of development, after which the mixtures were separated by SDS-PAGE. Figure 3.6.1B shows that a ³²P-labeled band that co-migrated with MBP-BldG was detected when MBP-BldG was incubated with *S. coelicolor* extract, and not when the extract

Figure 3.6.1 *In vitro* phosphorylation of MBP-BldG. Crude extracts (15 μ g total protein) isolated from *S. coelicolor* J1501 and Δ bldG 1DB plate cultures at various times (hours post-inoculation) as indicated were incubated at room temperature with purified MBP-BldG (1.5 μ g) in the presence of 5 μ Ci γ - ^{32} P-ATP. Reactions were terminated by the addition of SDS-PAGE sample buffer, and after separation by SDS-PAGE, protein bands were visualized by Coomassie staining (A) while protein phosphorylation was detected by exposing the gel to a phosphorscreen (B). Kaleidoscope pre-stained standards were used as the molecular weight marker, the sizes of which are indicated, as well as the position of MBP-BldG.

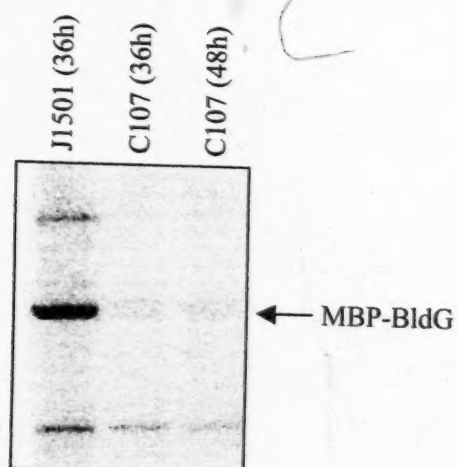


alone was separately incubated with γ - ^{32}P -ATP. Other ^{32}P -labeled bands that were observed in each lane were attributed to the phosphorylation of other unknown proteins in the *S. coelicolor* crude extracts, since these bands were routinely observed in the absence of added MBP-BldG. When similar reactions were performed using the purified untagged BldG protein (see Section 3.4.2), no phosphorylation of BldG could be detected (not shown). This was likely because the purified protein was partially or completely denatured, since the cleavage reaction to remove the MBP tag was carried out in the presence of SDS. Phosphorylation levels of MBP-BldG were consistently highest in extracts isolated during late vegetative growth (18h, Figure 3.6.1A), and when aerial mycelium and pigmented antibiotic were both visible (24h, 36h), and were found to drop off once sporulation had begun (48h). Interestingly, phosphorylated MBP-BldG was barely detectable when the purified protein was incubated with extract isolated from the *bldG* deletion mutant, and similar results were observed when extracts from the *bldGC107* mutant strain were used (Figure 3.6.2A). Since a high level of MBP-BldG phosphorylation could be restored by the presence of wild type BldG protein in the $\Delta bldG$ 1DB mutant extract (Figure 3.6.2B), this suggests that the kinase responsible for phosphorylation is somehow dependent on the presence of active BldG protein.

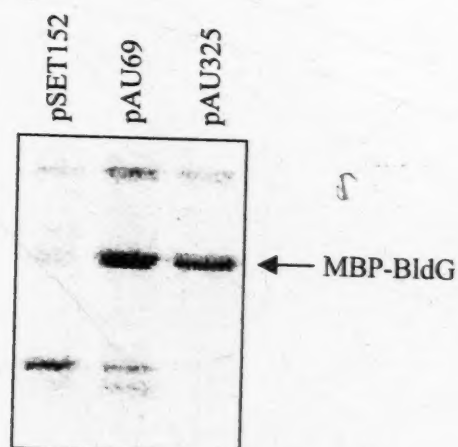
To determine which nucleotide serves as the phosphate donor for BldG, competition assays were performed in which purified MBP-BldG was incubated with crude extract and an excess of either cold ATP, CTP, GTP or UTP for 1 hour prior to the addition of γ - ^{32}P -ATP. The idea behind this assay is that if BldG is first incubated with the cold nucleotide that serves as the phosphoryl group donor, the phosphorylation sites will become occupied with a phosphoryl group, and therefore further incubation with γ -

Figure 3.6.2 (A) *In vitro* phosphorylation of MBP-BldG using extract isolated from *S. coelicolor* J1501 and from the *bldGC107* mutant strain. Crude extracts (15 μ g total protein) isolated from 36 or 48 hour plate cultures were incubated with purified MBP-BldG (3 μ g) at room temperature in the presence of 5 μ Ci of γ - ^{32} P-ATP. Reactions were terminated by the addition of SDS-PAGE sample buffer, then separated by SDS-PAGE, and protein phosphorylation was visualized by exposing the gel to a phosphorscreen. The position of MBP-BldG is indicated. (B) *In vitro* phosphorylation of MBP-BldG using extract isolated from the Δ *bldG* 1DB mutant strain containing either pSET152 (vector control), pAU69 (containing wild type *bldG*) or pAU325 (containing *bldGS57A*). Purified MBP-BldG (3 μ g) was incubated at room temperature in the presence of 5 μ Ci γ - ^{32} P-ATP with extract (15 μ g) isolated 48 hours post-inoculation from surface-grown cultures. Reactions were then processed, and protein phosphorylation was visualized as described above in (A). The position of MBP-BldG is indicated.

(A)



(B)



^{32}P -ATP will result in reduced (or no) ^{32}P -labeling of the protein. Figure 3.6.3 shows that the ^{32}P -labeling of MBP-BldG was competitively inhibited in this manner by an excess of ATP, whereas addition of excess CTP, GTP or UTP had no significant effect, indicating that ATP serves as the phosphate donor. To rule out the possibility that the ^{32}P -labeling of MBP-BldG was due to adenylation or non-specific binding of the γ - ^{32}P -ATP, rather than phosphorylation of BldG, *in vitro* phosphorylation assays were performed in which γ - ^{32}P -ATP was replaced with α - ^{32}P -ATP. As shown in Figure 3.6.3, ^{32}P -labeling of MBP-BldG did not occur when α - ^{32}P -ATP was substituted for γ - ^{32}P -ATP.

3.6.2 Isoelectric focusing

If phosphorylation of BldG occurs *in vivo* in *S. coelicolor*, the resulting modification would be expected to alter the isoelectric point of the protein such that it could be separated from the unphosphorylated form by isoelectric focusing. To further investigate this, *S. coelicolor* J1501 cell extracts isolated at various times during development (15-48 hours post-inoculation) were separated on a non-denaturing precast IEF gel which is able to resolve proteins that have a pI between 4.6 and 7.8. After transfer to PVDF membrane, anti-MBP-BldG antibodies were hybridized with the membrane in order to detect BldG and phosphorylated BldG (P-BldG) in the extracts. Figure 3.6.4 shows that two distinct bands were detected with the anti-MBP-BldG antibodies when the *S. coelicolor* J1501 crude extracts were separated by isoelectric focusing, and not when extract from the ΔbldG 1DB mutant was used (see Figure 3.3.6, ΔbldG 1DB+pSET152). Similar results were also obtained when extracts isolated from *S. coelicolor* M145 were used for isoelectric focusing (see Figure 3.6.6). When

Figure 3.6.3 Effect of nucleotide addition and α - ^{32}P -ATP on the phosphorylation of MBP-BldG. Purified MBP-BldG (0.15 μg) was incubated with 36 hour *S. coelicolor* J1501 crude extract (15 μg protein) and 1 mM of either ATP, GTP, CTP or UTP for 1 hour prior to the addition of 5 μCi of γ - ^{32}P -ATP. Control lanes include cell extract alone (no MBP-BldG or nucleotides) and cell extract with MBP-BldG and no excess cold nucleotide addition. For examining the effect of α - ^{32}P -ATP on phosphorylation, crude extract (36 hours, 15 μg protein) was incubated with MBP-BldG (1.5 μg) and 5 μCi of α - ^{32}P -ATP. Reactions were terminated by the addition of SDS-PAGE sample buffer and were separated by SDS-PAGE, and protein phosphorylation was visualized by exposing the gel to a phosphorscreen. The position of MBP-BldG is indicated.

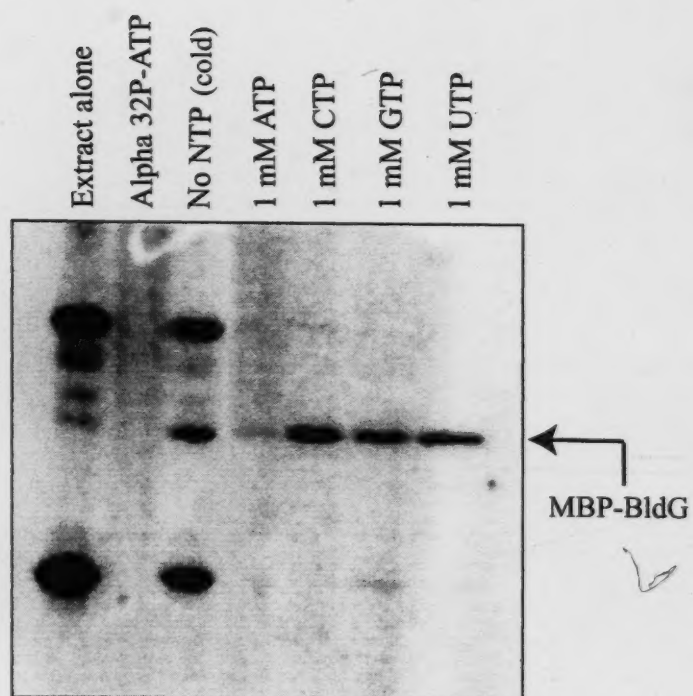
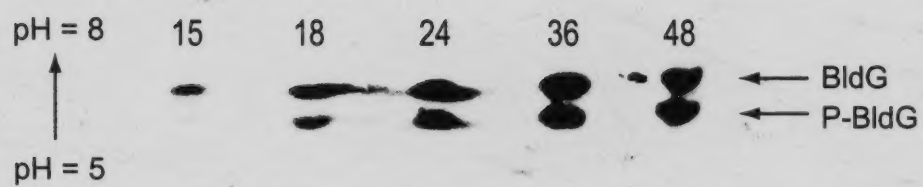


Figure 3.6.4 Detection of BldG and phosphorylated BldG by isoelectric focusing and western immunoblotting. Extracts (40 μ g) isolated from *S. coelicolor* J1501 at various times (hours post-inoculation) were mixed with 50% glycerol and were separated by isoelectric focusing on a non-denaturing precast IEF gel (pH = 5-8). After transfer to PVDF membrane, the extracts were probed with anti-MBP-BldG antibodies at a dilution of 1 in 20 000, followed by horseradish peroxidase-conjugated secondary antibody at a dilution of 1 in 10 000. Visualization of the bands was by incubating the membrane in Enhanced Plus chemiluminescence reagent, followed by exposure to film for 1-60 minutes. The direction of the pH gradient in the gel as well as the positions of the two forms of BldG are indicated.



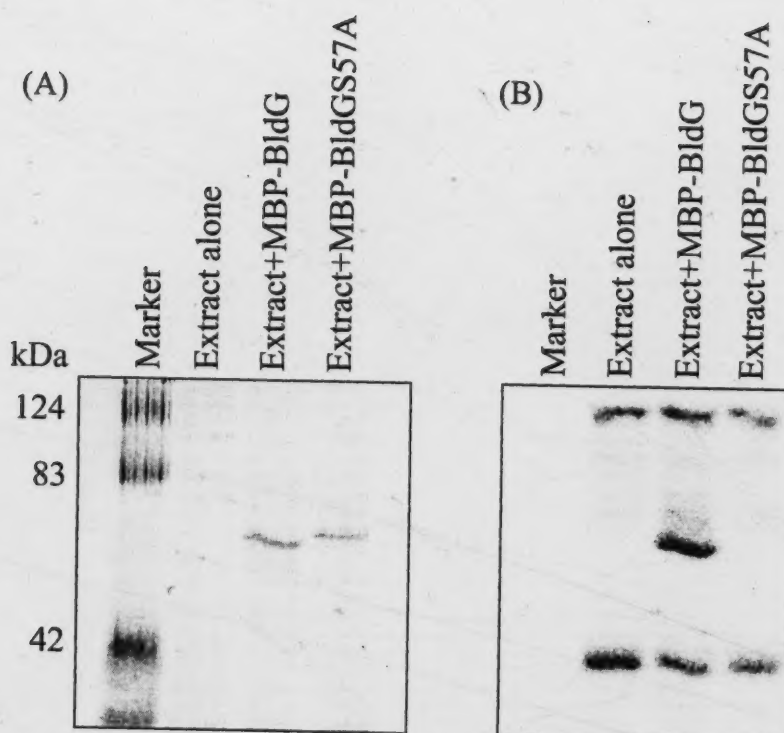
isoelectric points for BldG and P-BldG were calculated based on the amino acid composition, the pI values (4.69 and 4.47, respectively) indicated that the lower band would represent P-BldG while the upper band would represent BldG protein.

Densitometric analysis of the two bands revealed that during vegetative growth, the non-phosphorylated form of BldG is present in excess (6.4× at 15h, 2.7× at 18h), whereas once aerial hyphae and pigmented antibiotic synthesis begins (24h), the two forms are present in about equal amounts.

3.6.3 Effect of the S→A mutation at position 57 on the phosphorylation of BldG

Mutational studies revealed that the *bldGS57A* allele was unable to complement the bald phenotype of the $\Delta bldG$ 1DB null mutant strain, suggesting that the S57 residue is important for BldG stability and/or function (see Section 3.2.4). Western analysis of extract isolated from the $\Delta bldG$ 1DB strain containing either pAU69 (containing wild type *bldG*) or pAU325 (containing *bldGS57A*) demonstrated that the mutant BldGS57A protein could be detected at levels similar to wild type BldG protein levels, indicating that the S57A mutation does not affect protein stability (Figure 3.5.2B). Since the S57 residue corresponds to the phosphorylation site of SpoIIAA and RsbV, it was hypothesized that this residue is required for BldG phosphorylation, and that mutating it to an alanine residue would abolish phosphorylation, since alanine cannot serve as a substrate for phosphorylation. To examine this, *in vitro* phosphorylation assays were performed in which purified MBP-BldGS57A was incubated with *S. coelicolor* crude extract and γ -³²P-ATP, after which the reactions were separated by SDS-PAGE. Figure 3.6.5 shows that under the conditions used, phosphorylation of the wild type BldG

Figure 3.6.5 Effect of a S→A substitution at position 57 on the phosphorylation of BldG. Purified MBP-BldG or MBP-BldGS57A (1.5 µg) was incubated at room temperature with 36 hour *S. coelicolor* J1501 crude extract (15 µg protein) in the presence of 5 µCi γ -³²P-ATP. Extract without fusion protein was used as a negative control. Reaction components were separated by SDS-PAGE, and total protein was visualized by Coomassie staining (A) while protein phosphorylation was detected by exposing the gel to a phosphorscreen (B). Kaleidoscope pre-stained standards was used as the molecular weight marker, and the marker band sizes (in kDa) are indicated on the left.



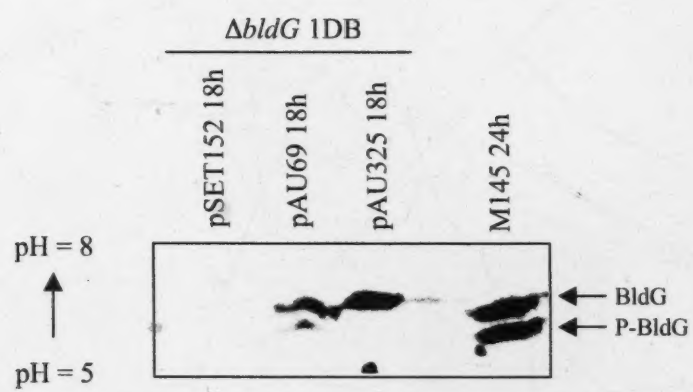
protein could easily be detected, however ^{32}P -labeling of the mutant protein was not observed, suggesting that S57 is the sole phosphorylation site in BldG. The effect of the S $\rightarrow$ A mutation was also investigated by isoelectric focusing, in which extract isolated from the ΔbldG 1DB strain containing either pAU69 or pAU325 was separated on a non-denaturing precast IEF gel, and was then analyzed by western immunoblotting using anti-MBP-BldG antibodies. As shown in Figure 3.6.6, the phosphorylated BldG protein could not be detected in the strain containing pAU325, confirming that the mutated serine residue is the phosphorylation site in BldG.

3.6.4 Effect of phosphorylation on BldG activity

If phosphorylation serves to regulate the activity of BldG in a manner similar to that observed for the *B. subtilis* SpoIIAA and RsbV proteins, then it would be expected that the BldGS57A mutant protein, which cannot be phosphorylated, is constitutively active. Since it was demonstrated that the *bldG*-specific kinase is partially dependent on the presence of active BldG protein, an assay was performed to determine whether extracts containing BldGS57A would be able to phosphorylate MBP-BldG *in vitro*. As shown in Figure 3.6.2B, the BldG-specific kinase is significantly more active in extract isolated from the ΔbldG 1DB strain containing pAU325 than in the control extract (ΔbldG 1DB + pSET152), suggesting that BldGS57A is active, and that the unphosphorylated form of BldG is most likely the active form of the protein.

The results of these experiments, therefore, indicate that BldG can be phosphorylated by a component of the *S. coelicolor* crude extract, and that this

Figure 3.6.6 Analysis of crude extracts from *S. coelicolor* M145 and $\Delta bldG$ 1DB by isoelectric focusing and western immunoblotting. The $\Delta bldG$ 1DB mutant strain contained either the pSET152 control plasmid, pAU69 (pSET152 containing wild type *bldG* gene) or pAU325 (pSET152 containing the *bldGS57A* mutant allele). Crude extract (40 μ g) isolated from 18 or 24 hour plate cultures were separated by isoelectric focusing on a non-denaturing precast IEF gel and were transferred to PVDF membrane. The membrane was then probed with anti-MBP-BldG antibodies at a dilution of 1 in 10 000, followed by horseradish peroxidase-conjugated secondary antibody at a dilution of 1 in 10 000, and bands were visualized by incubating with Enhanced Plus chemiluminescence reagent followed by exposure to film. The direction of the IEF pH gradient as well as the positions of BldG and P-BldG are indicated.



phosphorylation, which is somehow partially dependent upon BldG itself, is involved in the regulation of BldG activity. As well, the serine residue at position 57 appears to be the sole phosphorylation site in BldG, which is similar to what is observed with the *Bacillus* anti-anti-sigma factors. Since the BldGS57A mutant protein was unable to be phosphorylated, and the *bldGS57A* allele was unable to restore the wild type phenotype to the $\Delta bldG$ 1DB mutant strain, this suggests that phosphorylation of BldG is necessary for proper development in *S. coelicolor*.

3.7 Protein Binding Studies

The fact that the *bldG* locus shares a similar organization to that of the *spolIIA* and *rsb* loci of *B. subtilis* (except for the absence of a sigma factor gene), and the fact that *bldG* and *orf3* are co-transcribed as a polycistronic transcript suggested that the two proteins might work together to regulate a target protein, such as a sigma factor. If the proteins function in a manner analogous to their *Bacillus* counterparts, the ORF3 protein would presumably interact with its target protein to inhibit its activity, and this inhibition would be relieved upon BldG binding to ORF3, resulting in release of the target protein. In order to validate such an idea, however, it would be useful to demonstrate that BldG and ORF3 are able to form a complex. *In vitro* protein binding studies were therefore undertaken, including affinity chromatography and chemical cross-linking, and were performed using purified fusion proteins and/or BldG and ORF3 proteins overexpressed in *S. coelicolor*, in order to determine whether BldG and ORF3 are able to interact with one another.

3.7.1 Affinity chromatography

Affinity chromatography was chosen as the first method to look at protein-protein interactions, since it is quick, easy to perform, and it is very sensitive (Phizicky & Fields, 1995). The initial experimental strategy involved binding purified MBP-BldG to amylose resin, and then adding an equal amount of purified His₁₀-ORF3 and allowing the mixture to incubate. As a control, His₁₀-ORF3 was also incubated with resin which had no MBP-BldG bound to it. The resin was then washed with buffer, and if His₁₀-ORF3 were able to interact with BldG, it would remain associated with the amylose-bound MBP-BldG without being completely washed off. The bound proteins were then recovered from the resin by the addition of 10 mM maltose, and were analyzed by SDS-PAGE followed by western immunoblotting using anti-MBP-BldG and anti-His₁₀-ORF3 antibodies. Figure 3.7.1 shows that while MBP-BldG could be recovered from the amylose resin, His₁₀-ORF3 was not detected in the eluate. One possible explanation for this is that the binding reaction requires one or more additional factors in order for protein interaction to occur. To see whether this was the case, a couple of modifications to the original experiment were introduced. In one case, amylose-bound MBP-BldG was incubated with purified His₁₀-ORF3 in the presence of 500 μ M of various nucleotides (ATP, CTP, GTP, UTP, ADP), since it has previously been shown that the binding of SpoIIAA to SpoIIAB was enhanced by the presence of ADP (Alper *et al.*, 1994). In a second experiment, *S. coelicolor* crude extract (400 μ g, isolated at 18 and 36 hours post-inoculation) was included along with MBP-BldG and His₁₀-ORF3 in the binding reaction, since it was possible that the binding of ORF3 to BldG required some component normally present in *S. coelicolor* that was absent from the original *in vitro* system.

Figure 3.7.1 Affinity chromatography using purified MBP-BldG and His₁₀-ORF3.

MBP-BldG (3 µg) in 200 µl amylose column buffer containing 1× Complete EDTA-free protease inhibitor (Roche) was mixed with 50 µl of a 50% amylose slurry for two hours at 4 °C. The resin was washed with 3 × 1 ml of column buffer, resuspended in 200 µl column buffer + protease inhibitor, and mixed with purified native His₁₀-ORF3 (3 µg). As a control, His₁₀-ORF3 was also mixed with resin that had no MBP-BldG bound to it. The mixtures were incubated at 4 °C with gentle agitation for 14-16 hours, after which the resin was washed 3-5× with 1 ml of column buffer. Proteins bound to the resin were eluted by incubating the resin in column buffer containing 10 mM maltose for 10 minutes on ice, and then pelleting the resin and transferring the supernatant to a clean tube. The presence of BldG and ORF3 in the supernatant was then determined by SDS-PAGE and western immunoblotting using anti-MBP-BldG and anti-His₁₀-ORF3 antibodies. Purified MBP-BldG and His₁₀-ORF3 that did not go through the procedure were included as positive controls for protein detection.

+ His₁₀-ORF3

Resin + MBP-BldG

Resin - MBP-BldG

MBP-BldG

His₁₀-ORF3



employed. Neither of these modifications, however, had any effect on the results, and thus under the conditions used, interaction between purified MBP-BldG and His₁₀-ORF3 could not be demonstrated.

Another possibility for the inability to detect protein interaction is that the 10x histidine tag on ORF3 interfered with its ability to bind to BldG. Affinity chromatography experiments were therefore performed which attempted to pull out wild type ORF3 protein from *S. coelicolor* crude extracts. Amylose-bound MBP-BldG was incubated with *S. coelicolor* crude extracts (400-500 µg) isolated over a period of 15-48 hours post-inoculation from plate cultures, and proteins eluted from the resin were analyzed by western immunoblotting as described above. The experiment was also performed such that free MBP-BldG was first incubated with the crude extracts, and then amylose resin was added to the mixture to pull down the MBP-BldG and any proteins that were complexed with it. Unfortunately, both methods failed to pull out ORF3 from the extracts. Since it is possible that the level of ORF3 protein present in the extract was insufficient to be able to detect under the conditions used, the experiment was repeated using *S. coelicolor* extracts in which ORF3 was overexpressed from the thiostrepton-inducible *ptipA* promoter. Liquid cultures of *S. coelicolor* M600 containing pAU317 (see Table 2.4) were grown to an OD₄₅₀ ~1, and were then induced with thiostrepton (30 µg/ml final concentration). The induced cultures were incubated for 2 hours at 30 °C, and extracts were prepared using a gentle lysis method in which protoplasts were generated from the induced mycelia, and were then lysed in low osmotic buffer. The resulting extract was then incubated with MBP-BldG in column buffer containing protease inhibitors, and samples were taken before and after the addition of amylose

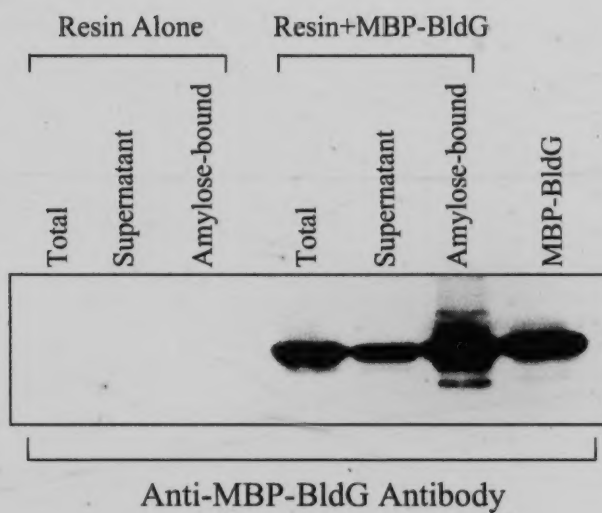
resin. Proteins bound to the resin were eluted by adding SDS-PAGE sample buffer to the resin and boiling the sample for 5-10 minutes. Figure 3.7.2 shows that ORF3 protein could be detected by western analysis in the total protein mixture before amylose addition, and in the supernatant after amylose addition, however only a trace amount could be detected in the MBP-BldG-containing sample eluted from the resin. Since an equivalent amount of ORF3 could also be detected in the sample eluted from the amylose resin to which no MBP-BldG was attached, it was concluded that the ORF3 protein detected in these samples was due to inefficient washing of the resin, or to non-specific interaction of the protein with the resin.

3.7.2 Chemical cross-linking experiments

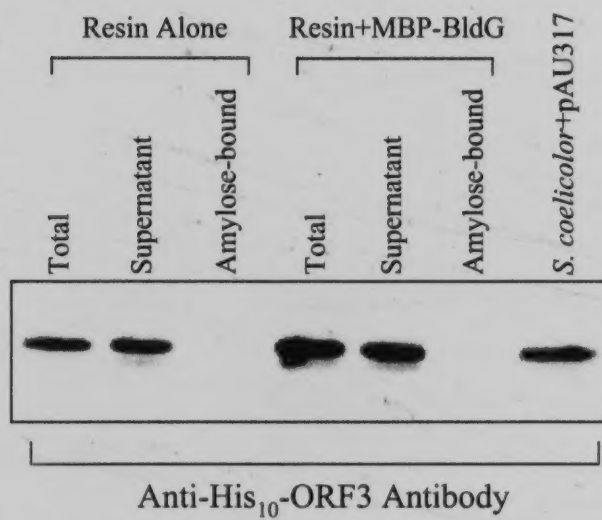
The inability to detect complex formation between BldG and ORF3 by affinity chromatography may have been due to a couple of reasons. First, it was assumed when performing these experiments that the presence of the MBP-tag on BldG was not significantly affecting its ability to undergo protein interactions, since it had been demonstrated that the fusion protein could be phosphorylated by a kinase present in *S. coelicolor* crude extracts (see Section 3.6.1). Interaction with a kinase, however, would most likely occur transiently, and therefore may not be affected by the presence of the tag to the same extent as a more stable interaction. The size of the MBP-tag is significantly larger than that of BldG (42 kDa versus 12.3 kDa), and therefore the tag could in-fact be interfering with the ability of BldG to form a stable complex with ORF3. Secondly, the interaction between BldG and ORF3 may be fairly weak such that it cannot be detected

Figure 3.7.2 Affinity chromatography using purified MBP-BldG and *S. coelicolor* crude extract containing overexpressed ORF3 protein. MBP-BldG (10 μ g) was mixed in amylose column buffer containing protease inhibitors with extract (~400 μ g total protein) isolated from *S. coelicolor* M600 + pAU317 which had been induced with thiostrepton, and the mixture was incubated at 4 °C for 16 hours. As a control, crude extract was also incubated in column buffer in the absence of MBP-BldG. A sample of the total protein (10 μ l) was then taken from each tube and was mixed with 5 μ l of SDS-PAGE sample buffer, after which amylose resin (50 μ l of a 50% slurry in column buffer) was added to each tube, and the tubes were allowed to incubate with gentle shaking for 2 hours at 4 °C. The resin was pelleted, a 10 μ l sample of the supernatant was taken and mixed with SDS-PAGE sample buffer, and the resin was washed with 3 $\times$ 1 ml of column buffer. Proteins bound to the resin were then eluted by adding SDS-PAGE sample buffer and boiling the tube for 5-10 minutes. Samples were separated by SDS-PAGE on a 10 or 12% polyacrylamide gel, and after transfer to PVDF membrane, were probed separately for MBP-BldG (A) and ORF3 (B). Purified MBP-BldG and extract containing ORF3 that did not go through the procedure were included as positive controls for protein detection.

(A)



(B)

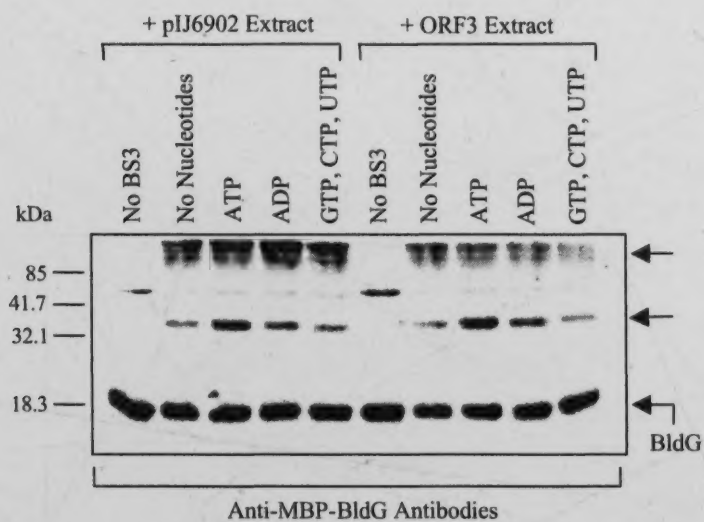
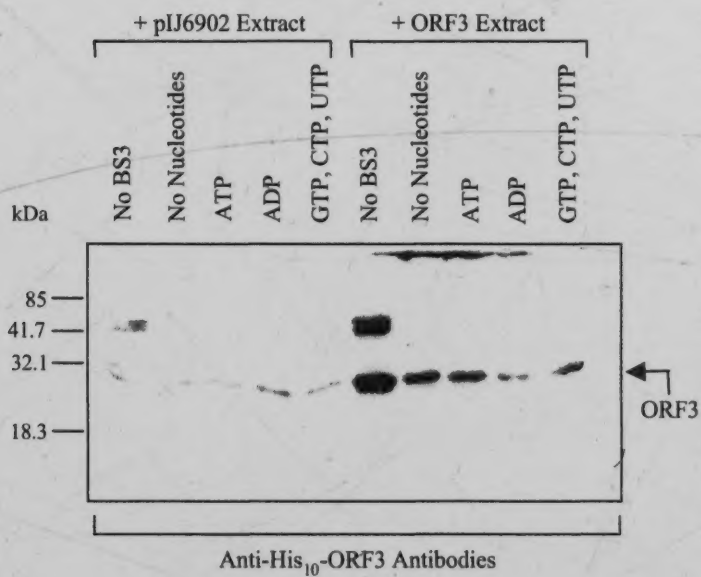


using methods such as affinity chromatography, which rely on the proteins remaining associated during various incubation and wash steps.

To address both of these possibilities, chemical cross-linking reactions, which like affinity chromatography are quick and easy to perform with the added advantage of being able to detect weak interactions, were next performed using untagged BldG and ORF3 proteins. The BldG overexpression plasmid pAU316 was constructed by PCR amplification of a 483 bp fragment using pAU64 as the template and the primers DBG35 and DBG3 (see Table 2.5). The resulting product was purified, digested with *Nde*I and *Xba*I, and then ligated into similarly-digested pIJ6902, and the resulting plasmid was introduced into *S. coelicolor* $\Delta bldG$ 1DB by conjugation. As in the case of the *orf3* gene cloned into pAU317 (see Section 3.2.7), expression of *bldG* in pAU316 was under control of the thiostrepton-inducible promoter *ptipA*, and extracts were isolated from induced cultures of $\Delta bldG$ 1DB containing either pAU316 or pAU317 using the gentle lysis method described above (Section 3.7.2). As a control, the same procedure was performed using a *S. coelicolor* $\Delta bldG$ 1DB strain that contained the plasmid vector (pIJ6902) alone. The basic cross-linking experiment involved incubating together equivalent amounts of *S. coelicolor* extract containing either overexpressed BldG or ORF3 protein in binding buffer to allow complex formation to occur, and then treating the mixture with BS3 cross-linker (1 mM final concentration) in order to covalently trap any complexes that had formed. BldG and ORF3-containing complexes could then be detected by subjecting the reactions to SDS-PAGE followed by western immunoblotting using anti-MBP-BldG and anti-His₁₀-ORF3 antibodies. Initial experiments revealed the formation of a BldG-containing complex with a size of about 35 kDa when BS3 was

added to the reaction, however this same complex was not detected when ORF3-specific antibodies were used for western analysis. The experiment was repeated in which extract containing overexpressed BldG was mixed with the control extract (pIJ6902) or with the overexpressed ORF3 extract in the presence and absence of ATP, ADP, CTP, GTP or UTP, since as mentioned previously, complex formation may be enhanced by the presence of these nucleotides. Figure 3.7.3 shows that when overexpressed BldG extract was mixed with either the control extract or the overexpressed ORF3 extract in the presence of ATP, higher levels of the 35 kDa complex were observed compared to when no nucleotides were added. Increased levels of the complex were also observed when ADP was added, although to a lesser extent than when ATP was used. As observed with the previous cross-linking experiment, ORF3-specific antibodies did not react with a similar-sized complex, suggesting that the 35 kDa complex does not involve ORF3. This was also suggested by the fact that the amount of the complex formed did not vary with different amounts of ORF3 protein present (compare the control lanes with the ORF3-containing lanes). Interestingly, the BldG-specific antibodies also detected higher molecular weight complexes (>85 kDa) that were only present when the BS3 cross-linker was added and that were also not detected by the ORF3-specific antibodies. A third band was also detected on the BldG western, although this band most likely represent a cross-reacting protein, since the same band was detected in extracts isolated from the $\Delta bldG$ 1DB strain containing pIJ6902, where no BldG protein of the correct molecular weight was detected (not shown).

Figure 3.7.3 Chemical cross-linking reactions using *S. coelicolor* extracts containing overexpressed BldG and ORF3. Extracts (20 μ g) isolated from the *S. coelicolor* Δ bldG 1DB strain containing either pAU316 or pAU317 for overexpression of BldG or ORF3, respectively, were incubated together in binding buffer in the presence or absence of 500 μ M of ATP, ADP, GTP, CTP or UTP. Similar reactions were also set up in which overexpressed BldG extract was incubated with extract from *S. coelicolor* Δ bldG 1DB containing the plasmid vector alone (pIJ6902). BS3 cross-linker (1 mM) was then added, and after incubation for 30 minutes, the reactions were terminated by the addition of lysine (20 mM). Reactions in which no BS3 was added were also included as controls. Samples were then separated by SDS-PAGE, transferred to PVDF, and probed separately with BldG or ORF3 antibodies. Kaleidoscope pre-stained standards was used as the molecular weight marker, the band sizes of which are indicated (in kDa). The positions of the BldG and ORF3 proteins are indicated, as well as the positions of the 35 kDa and the higher molecular weight BldG-containing complexes (indicated by $\leftarrow$).

S. coelicolor $\Delta bldG$ 1DB/pAU316 Extract*S. coelicolor* $\Delta bldG$ 1DB/pAU316 Extract

The results of these experiments, therefore, indicate that BldG can be cross-linked to some protein (or proteins) in *S. coelicolor* crude extract that may be interacting with BldG, however this protein does not appear to be the ORF3 putative anti-sigma factor.

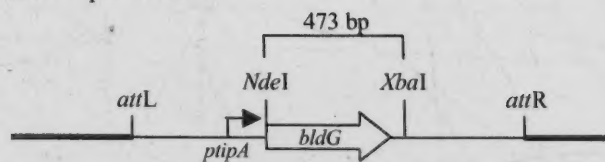
3.8 DNA Microarray Analysis

The availability of the *S. coelicolor* genome sequence as well as advances in DNA microarray technology have made it possible to assess simultaneously global changes in gene expression in *Streptomyces* in response to certain factors. We wanted to use DNA microarrays to identify genes whose transcription was affected by BldG and ORF3, in hopes of using this information to identify a putative sigma factor that may be regulated by the two proteins. Experiments were performed that looked at changes in global transcription profiles in response to overexpression of BldG or ORF3 in the wild type strain, and in response to overexpression of BldG in the *bldGC103* mutant background. As well, the effect of *bldG* on gene expression was assessed by comparing the global transcription patterns in the wild type strain and in the *bldGC103* mutant strain during development. This work was carried out in collaboration with Dr. Camilla Kao (Stanford University, USA), and experiments involving the microarrays themselves were performed in Stanford by either Dr. Brenda Leskiw or by Nitsara Karoonuthaisiri of Dr. Kao's lab.

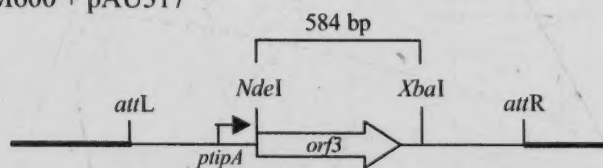
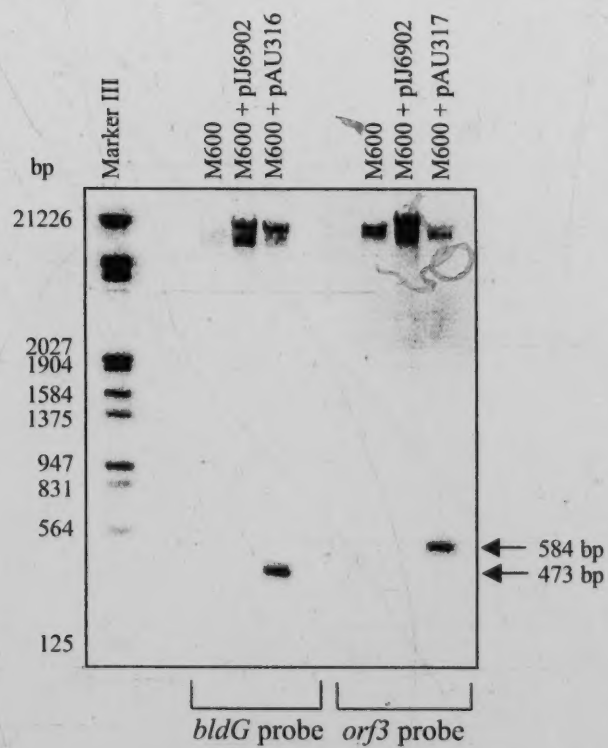
3.8.1 Analysis of global gene expression in response to overexpression of BldG and ORF3 in wild type *S. coelicolor*

The effect of BldG and ORF3 on gene expression in *S. coelicolor* was first examined by overexpressing the two proteins separately in the wild type strain. If ORF3 does in-fact function as an anti-sigma factor that sequesters a sigma factor in an inactive complex, and BldG counteracts this inhibition by binding to ORF3, then it is thought that the overexpression of each protein would have opposing effects on the transcription of genes under control of the BldG/ORF3-regulated sigma factor. For example, the overexpression of ORF3 would be predicted to cause repression of the sigma factor-targeted genes, since the sigma factor would be bound to and inhibited by the excess ORF3 protein that is present. On the other hand, if BldG were overexpressed, transcription of the target genes would be expected to be induced, since the excess BldG protein would compete with the sigma factor for binding to ORF3, which would presumably lead to the accumulation of free sigma factor protein. Construction of the BldG and ORF3 overexpression vectors (pAU316 and pAU317, respectively), where *bldG* and *orf3* were under control of the thiostrepton-inducible *ptipA* promoter, was described previously (Sections 3.2.7 and 3.7.2), and both plasmids were introduced into *S. coelicolor* M600 by conjugation from *E. coli* ET12567/pUZ8002. The plasmids were maintained in *Streptomyces* by integration into the chromosome at the Φ C31 *attB* site, and verification of plasmid integration was by Southern analysis (Figure 3.8.1). As a control, the parent vector (pIJ6902) was also introduced into *S. coelicolor* M600, so that thiostrepton-induced changes in gene expression could be accounted for.

Figure 3.8.1 Verification of pAU316 and pAU317 integration into the *S. coelicolor* chromosome. (A) Schematic diagram of M600 genomic DNA showing integration of pAU316 and pAU317 into the ϕ C31 *attB* site. The location of the *Nde*I and *Xba*I sites, which were engineered into the original PCR primers used to amplify *bldG* and *orf3* for cloning into pIJ6902, are shown. Digestion of the chromosomal DNA with *Nde*I and *Xba*I would result in release of a 473 bp *bldG*-containing fragment in the case of M600 + pAU316, and a 584 bp *orf3*-containing fragment in the case of M600 + pAU317, where *bldG* and *orf3* are indicated by the gray arrows. The M600 chromosomal DNA is represented by the thick black line whereas plasmid DNA is represented by the thin black line. Also indicated are the *attL* and *attR* sites, which result from recombination between the *attP* site on the plasmid and the chromosomal *attB* site, and the thiostrepton-inducible *ptipA* promoter used for expression of *bldG* and *orf3* (indicated by the black arrow). (B) The *Nde*I/*Xba*I-digested chromosomal DNA from M600 with the integrated pAU316 or pAU317 was separated by electrophoresis on a 1% agarose gel along with similarly-digested DNA from plasmid-free M600 and M600 + pIJ6902. Marker III (Roche) was included as the molecular weight marker, and after transfer to membrane, the marker lane was cut off and probed with ³²P-random primer labeled marker III DNA. The remaining samples were probed with either a ³²P-random primer labeled internal *bldG* fragment (BKL 63-64, see Section 3.3.1) or a ³²P-random primer labeled internal *orf3* fragment (DBG8-BKL83, see Section 3.3.1). The position of the 473 bp *bldG*-containing fragment from M600 + pAU316, and of the 584 bp *orf3*-containing fragment from M600 + pAU317 are shown, as well as the sizes (in bp) of the molecular weight marker bands. The upper bands in each lane represent the chromosomal copies of *bldG* and *orf3*.

A M600 + pAU316

M600 + pAU317

**B**

The setup for the overexpression experiment is shown in Figure 3.8.2. Spring flasks containing NMMP medium were inoculated with spores of the control strain (M600 + pIJ6902), the BldG overexpression strain (M600 + pAU316), or the ORF3 overexpression strain (M600 + pAU317) that had been pre-germinated in order to synchronize the growth. The flasks (six per strain) were incubated at 30 °C until the OD₄₅₀ was about 0.5, after which time the respective cultures were combined into a large single flask and redistributed into new spring flasks to minimize variations between the individual cultures. An aliquot of the culture was kept aside for the Time 0 (uninduced) sample, and the flasks were then induced with thiostrepton, and were incubated for various times before harvesting the mycelia for RNA isolation. The RNA was used in DNA microarray experiments, where the Time 0 samples (labeled green) were used as the references to which all subsequent samples (labeled red) were compared. In this manner, genes which became repressed in response to BldG or ORF3 overexpression appeared green on the arrays, whereas genes which became induced in response to BldG or ORF3 overexpression appeared red. The arrays used for the initial experiments contained ~5000 of the 7825 annotated protein-encoding genes of the *S. coelicolor* genome, whereas later experiments used arrays containing 7071 genes.

The results obtained with the BldG overexpression strain were largely uninformative, since it was found that the transcription profile of the represented genes on the arrays was no different than that observed for the control strain. When the RNA samples were probed by northern analysis with a DNA fragment specific for *bldG*, it was found that the basal level of *bldG* transcripts was already high (Figure 3.8.3), and

Figure 3.8.2 Schematic drawing of the overexpression experiments performed for DNA microarray analysis. Pre-germinated spores or mycelia from starter cultures were used to inoculate spring flasks containing 100 ml (for Time 0) or 50 ml (for all other samples) of NMMP or R2YE medium. The flasks were incubated at 30 °C until the OD₄₅₀ was ~ 0.5, and then the cultures were combined and redistributed into fresh spring flasks, keeping 100 ml aside for the Time 0 sample. Thiostrepton (30 µg/ml final concentration) was then added to each flask, and the flasks were incubated for 15, 30, 45, 60, and 120 minutes. For RNA isolation, mycelia were harvested from a single flask at each time point.

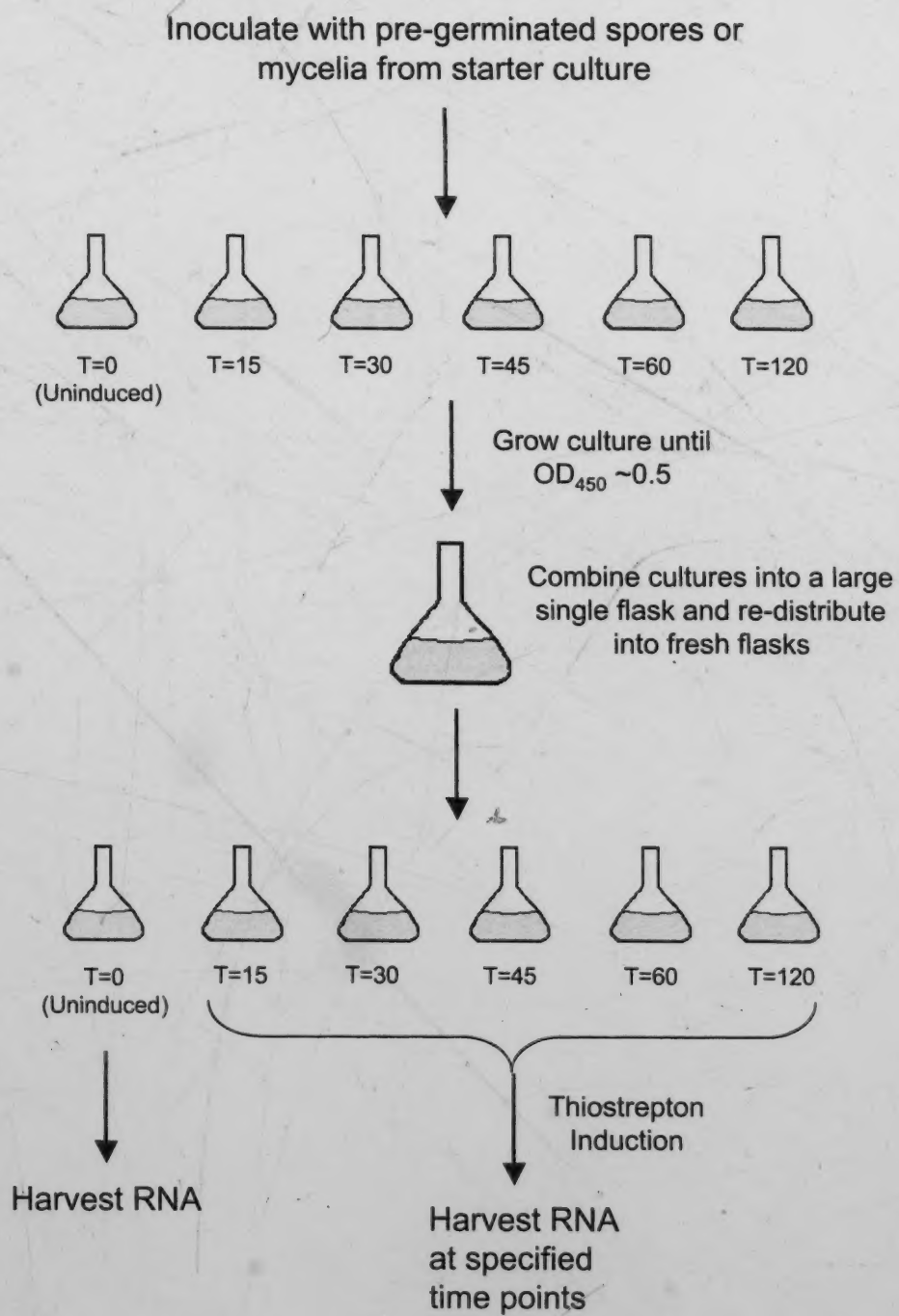
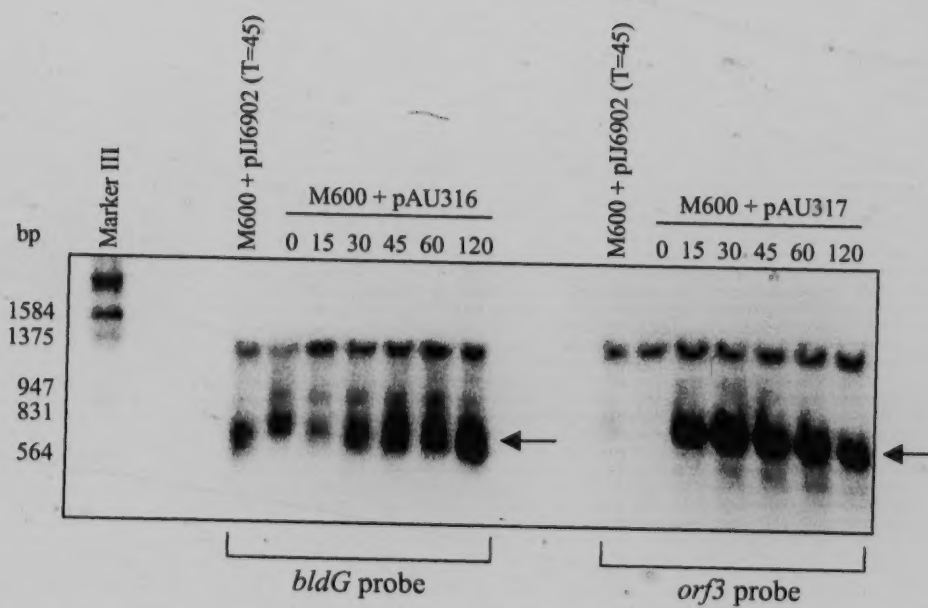


Figure 3.8.3 Northern analysis of *bldG* and *orf3* transcripts in thiostrepton-induced M600 + pAU316 and M600 + pAU317 strains. Total RNA isolated before (0 minutes) and after thiostrepton induction (15-120 minutes) from M600 + pAU316 or M600 + pAU317 was denatured along with Marker III (Roche) and separated on a 1.25% agarose gel. RNA isolated from M600 + pIJ6902 after thiostrepton induction (T = 45 minutes) was also included as a control. After transfer to membrane, the marker lane was cut off and probed separately with ^{32}P -random primer labeled Marker III, while the M600 + pAU316 samples were probed with a ^{32}P -random primer labeled internal *bldG* fragment (BKL 63-64), and the M600 + pAU317 samples were probed with a ^{32}P -random primer labeled internal *orf3* fragment (DBG8-BKL83). The induced *bldG* and *orf3* transcripts are indicated by the arrows, while the upper band in both blots represents the *bldG-orf3* polycistronic transcript. The sizes of the molecular weight marker bands are indicated in bp.



therefore inducing *bldG* expression with thiostrepton likely would not change the expression pattern of the target genes. A high basal level of *bldG* transcript was also observed by DNA microarray analysis in which *S. coelicolor* genomic DNA (labeled green) was compared to RNA isolated from an induced control sample (labeled red, data not shown). On the other hand, a large increase in *orf3* transcript levels was observed in RNA samples from the induced ORF3 overexpression strain compared to the uninduced sample and the control sample (Figure 3.8.3), and so we focused on these samples for DNA microarray analysis.

Table 3.1 lists the genes whose transcription was either repressed or induced more than two-fold at no less than three time points where ORF3 was overexpressed compared to the uninduced sample. A good portion of the genes that were affected encoded hypothetical proteins for which there is little information. Interestingly, a number of regulatory proteins were found to be affected by ORF3 overexpression, including ones belonging to the TetR family of transcription regulators (SCD95A.18c and SCI30A.20c), and one protein belonging to the MarR family of regulators (2SCG38.38), a family that includes proteins involved in the sensing of phenolic compounds (Sulavik *et al.*, 1995). Also affected were a number of genes encoding putative membrane proteins and secreted proteins, as well as genes encoding a putative anti-anti-sigma factor and a putative partitioning or sporulation protein. The most strongly affected genes (excluding *orf3* itself) were 2SCD60.20 (encoding a putative integral membrane protein) and SCI7.09c (encoding a hypothetical protein), which showed repression levels of about 7.5-8.1 fold and 2.8-4.3 fold, respectively, over three time points, while SC8F4.02c (encoding a



Table 3.1: DNA microarray data of genes whose transcription was affected by BldG and/or ORF3 overexpression

Gene Designation	Annotation	Transcription Induced/Repressed
Overexpression of ORF3 in <i>S. coelicolor</i> M600		
SCD8A.16c	Putative secreted protein	Repressed
SCD8A.22	Hypothetical protein	Repressed
SCE66.21	Hypothetical protein	Repressed
SCF6.08c	Putative regulatory protein	Repressed
SC4G10.04c	Putative anti-sigma factor antagonist	Repressed
SC4G1.30	Putative secreted protein	Repressed
SCI7.09c	Hypothetical protein	Repressed
2SCG38.38	Putative MarR-family regulator	Repressed
SCI1B5.06c	Hypothetical protein	Repressed
SCC80.04c	ATP-dependent Clp protease proteolytic subunit 1	Repressed
SCI30A.20c*	Putative transcriptional regulator (TetR family)	Repressed
SCE15.03c	Hypothetical protein (Putative secreted protein)	Repressed
SCC53.32c	Hypothetical protein	Repressed
2SCD60.20	Putative integral membrane protein	Repressed
SC5H4.22	Hypothetical protein	Repressed
SC2A11.07	Putative ATP/GTP binding protein	Repressed
SC3H12.13c	Hypothetical protein	Repressed
2SCD46.39	Putative integral membrane protein	Repressed
AfsS*	Global regulator of antibiotic production	Repressed
2SCK36.13*	Conserved hypothetical protein	Repressed
SCH5.11c (ORF3)	Putative anti-sigma factor	Induced
SCE46.04c	Putative regulatory protein	Induced
STH24.08	Putative partitioning or sporulation protein	Induced
ORF_1 (<i>whiE</i> locus)	Unknown	Induced
SC9E12.10c	Hypothetical protein	Induced
SCF76.18c	Putative membrane protein	Induced
SCF20.03	Hypothetical protein	Induced
SCD66.16	Hypothetical protein	Induced

Gene Designation	Annotation	Transcription Induced/Repressed
SC8F4.02c	Conserved hypothetical protein	Induced
SC6D10.20c	Putative ABC transporter (ATP-binding protein)	Induced
SCD95A.18c	Putative regulatory protein (TetR family)	Induced
SC1C2.18c	Putative ATP/GTP binding protein	Induced
SCE46.04c	Putative regulatory protein	Induced
SC6A9.29	Integral membrane protein	Induced
SC1A9.03	Putative sensor kinase	Induced
SC6A9.29	Integral membrane protein	Induced
Overexpression of BldG in		
<i>bldGC103</i> mutant strain		
SC5F2A.05c	Putative regulator	Induced
SC2H2.15	Hypothetical protein	Induced
SC130A.21c	Hypothetical protein	Induced
SC4B10.13c	ECF sigma factor	Induced
SC3C3.12	Hypothetical protein	Induced
SCF55.32*	Possible transcriptional regulatory protein	Induced
2SCK36.13*	Conserved hypothetical protein	Induced
AfsS*	Global regulator of antibiotic production	Induced

* Denotes genes which appear more than once in this Table and also appear in Table 3.2

† These genes were detected by screening the array data for genes whose transcription was affected by BldG overexpression in the C103 mutant strain

conserved hypothetical protein) and SC6A9.29 (encoding an integral membrane protein) displayed induction levels of 3.8-5.4 and 4.9-5.7 fold, respectively.

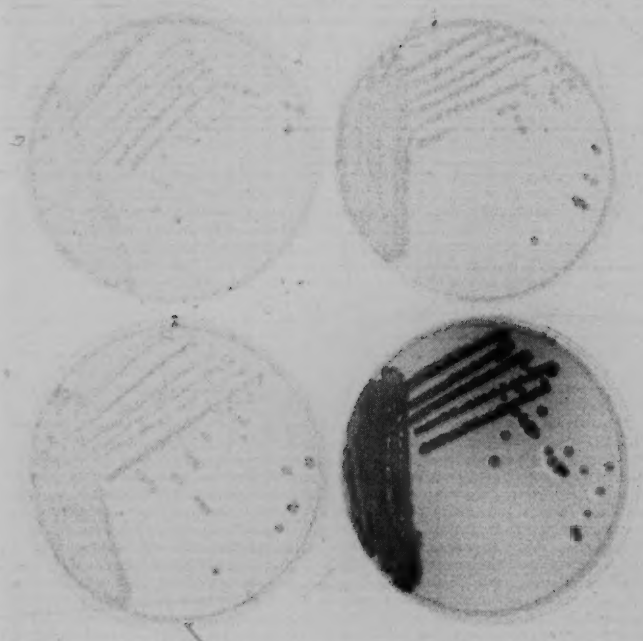
3.8.2 Analysis of global gene expression in response to overexpression of BldG in the *bldGC103* mutant strain

Since overexpression of BldG in the wild type strain did not yield any usable data, we next looked at performing experiments in which BldG was overexpressed in a *bldG* mutant background. The pIJ6902 (control) vector and the pAU316 BldG overexpression vector were both introduced by conjugation into *bldGC103* mycelial fragments from *E. coli* ET12567/pUZ8002, and the presence of the vectors in selected exconjugants was verified by growing the strains on R2YE plates in the presence of apramycin (for vector selection) and in the presence and absence of thiostrepton (for protein induction). The results, shown in Figure 3.8.4, indicate that complementation of the *bldG* mutant phenotype only occurred in the strain containing pAU316 and only when thiostrepton was present in the medium, confirming that the overexpressed BldG protein was functional. In addition, the lack of complementation in the absence of thiostrepton showed that expression from the *ptipA* promoter was not excessively leaky. Induction experiments were performed as described for overexpression of BldG and ORF3 in the wild type strain (see Figure 3.8.2), except that the flasks were inoculated with mycelia from starter cultures rather than pre-germinated spores (see Section 3.1.1). Also, since the mutant strain was unable to grow efficiently in NMMP medium, the experiments were performed using R2YE broth containing 6% PEG 8000 to enhance dispersal of the mycelia. RNA isolated at the various time points was then subjected to northern analysis

Figure 3.8.4 Growth of the *bldGC103* mutant strain containing either pIJ6902 or pAU316 in the presence and absence of thiostrepton. The strains were grown on R2YE agar for 36 hours at 30 °C. The production of aerial hyphae and of blue and red pigments by the pAU316-containing C103 strain grown in the presence of thiostrepton indicates complementation of the mutant phenotype by the presence of the BldG overexpression vector.

R2YE - Thiostrepton

R2YE + Thiostrepton



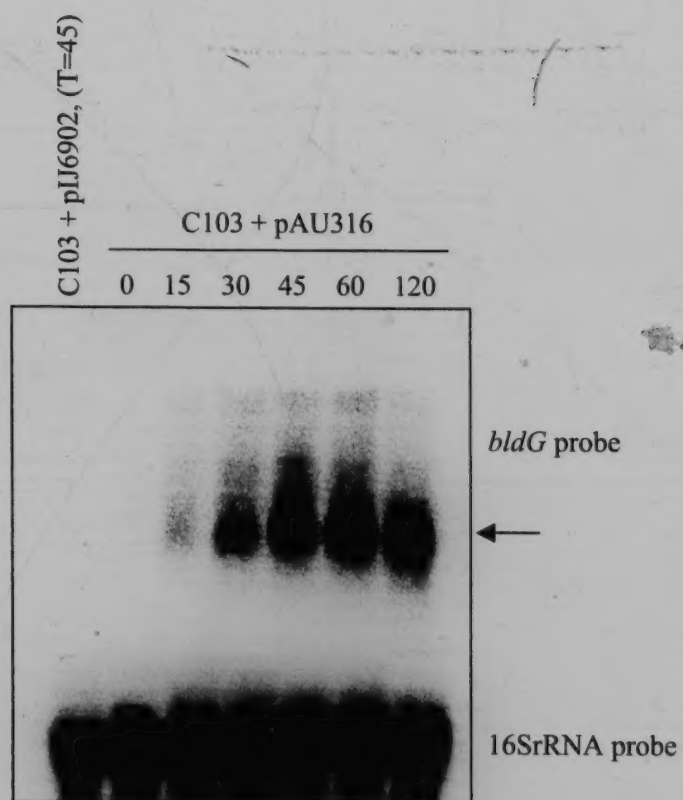
C103 + pIJ6902

C103 + pAU316

using an internal *bldG*-specific probe to verify that induction of *bldG* expression had taken place. As seen in Figure 3.8.5, the addition of thiostrepton resulted in a significant increase in *bldG* monocistronic transcript levels, confirming that induction had occurred.

The microarray experiments were performed in a similar manner to the ones performed using the ORF3-overexpressed samples, where the Time 0 (uninduced) sample was labeled with Cy-5 (green), and was used as the reference to which all subsequent samples (labeled red) were compared. Table 3.1 lists genes that were found to be induced more than two-fold in response to BldG overexpression at three different time points. Genes that appeared to be repressed in response to BldG overexpression were not represented on the table, since control data was not available for these genes. Among the proteins induced were an ECF sigma factor (showing 2.3-3.6 fold induction) and two putative regulatory proteins (SC5F2A.05c, showing 2.3-5.1 fold induction, and SCF55.32, showing 2.4-6.1 fold induction). Interestingly, the *afsS* gene, which as previously mentioned encodes a protein involved in the regulation of antibiotic production in *S. coelicolor* (see Section 1.4), was also induced ~4-6 fold in response to BldG overexpression. When the transcription profile of *afsS* and of the 2SCK36.13 gene was examined in the microarray data from the ORF3-overexpression experiments, both genes were found to be repressed in response to ORF3 overexpression. The opposing effects of BldG and ORF3 on *afsS* and 2SCK36.13 transcription suggests that these genes may represent targets of a BldG/ORF3-regulated sigma factor, since as previously mentioned, it is predicted that overexpression of ORF3 would result in sequestering of the target sigma factor (and hence repression of target genes) whereas overexpression of BldG would result in release of the target sigma factor (and hence induction of target

Figure 3.8.5 Northern analysis of *bldG* transcripts in the *bldGC103* mutant strain containing the inducible *bldG* overexpression plasmid pAU316. Total RNA isolated before (0 minutes) and after thiostrepton induction (15-120 minutes) was denatured along with control RNA isolated from *bldGC103* containing the pIJ6902 vector after thiostrepton induction (T = 45 minutes), and was separated on a 1.25% agarose gel. After transfer to membrane, the samples were probed with a ^{32}P -random primer labeled internal *bldG* fragment (BKL 63-64). To control for RNA loading levels, the same blot was probed with a ^{32}P -end-labeled oligonucleotide (BKL54, See Table 2.5) specific for 16S rRNA. The induced *bldG* transcript is indicated by the arrow.



genes). However, since the control data for the ORF3 overexpression experiment were not useful for either *afsS* or 2SCK36.13, the experiments would have to be repeated in order to verify that ORF3 overexpression does in-fact result in repression of these two genes.

3.8.3 Comparison of gene expression in the *bldGC103* mutant and in the *bldG*⁺ congenic strain J1501

A final experiment was performed which looked at global gene expression in the *bldGC103* mutant strain in comparison to the wild type *S. coelicolor* J1501 strain at various stages of development. Strains were grown at 30 °C on the surface of cellophane disks on R2YE plates for 15, 18, 24, 36 and 48 hours, and the biomass was harvested for RNA isolation at each time point. DNA microarray analysis was then performed using the RNA, where the earliest time point (15 hours) for each strain was labeled green, and was compared to all other time points (labeled red). Table 3.2 lists the genes whose expression pattern differed in the mutant strain compared to the wild type strain, while the expression pattern for most of these genes is shown in Figure 3.8.6. The *afsS* gene (also represented by SCD6.03c on the array) was found to be induced in the wild type strain at 18 and 36 hours compared to the 15 hour time point, whereas levels at 24 and 48 hours were comparable to that at 15 hours. In the mutant strain, however, the transcript levels at 18 hours were similar to the 15 hour time point, and expression was slightly repressed at all other time points examined (Figure 3.8.6). Interestingly, a similar transcription pattern was also observed for SCD84.08c, SCF55.32 and SCI30A.20c, where induction levels for all the genes in the wild type strain were generally about 4-6

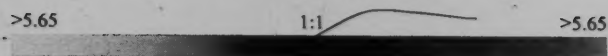
Table 3.2: DNA microarray data of genes whose transcription profile differed in the *bldGC013* mutant strain compared to the wild type J1501 strain

Gene Designation	Annotation
SCD6.03c (AfsS)*	Global regulator of antibiotic production
2SCK36.13*	Conserved hypothetical protein
SC2A11.10	Oligopeptide transport integral membrane protein
SC2A11.14	ABC oligopeptide transporter (ATP-binding)
SC2A11.12	Integral membrane protein (ABC transport system)
SC2A11.13	Oligopeptide transporter (ATP-binding)
SC2A11.11	Putative oligopeptide-binding lipoprotein
SCF55.32*	Possible transcriptional regulatory protein
SCE9.18c	Possible membrane protein
SC9A10.09	Hypothetical protein
2SC7G11.22c	Secreted protein
2SC7G11.18c	Hypothetical protein
SC130A.20c*	Putative transcriptional regulator (TetR family)
2SCD46.41c	Hypothetical protein
SCD84.08c	Putative Pst transport system integral membrane protein
SC44.02c	Hypothetical protein

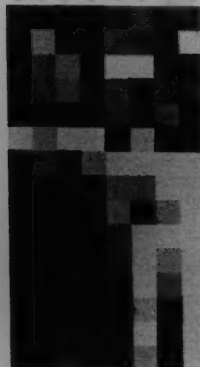
* Denotes genes which also appear in Table 3.1

Figure 3.8.6 Expression profile of genes whose expression differed in the *bldGC103* mutant strain compared to wild type J1501. Profiles indicate the extent of gene expression relative to the first sample taken (15 hour sample) for each strain, with red indicating an increase in transcription, green indicating a decrease in expression, and black representing similar expression levels relative to the reference. Gray coloring indicates that no data was available. Columns correspond to successive time points, whereas rows correspond to individual genes, the annotated products of which are indicated. Genes were clustered according to similarity in the expression profile.

Scale (fold repression or induction)



C103 15h vs C103 18h
C103 15h vs C103 24h
C103 15h vs C103 36h
C103 15h vs C103 48h
J1501 15h vs J1501 18h
J1501 15h vs J1501 24h
J1501 15h vs J1501 36h
J1501 15h vs J1501 48h



SCD84.08c	Putative Pst transport system integral membrane protein
SCI30A.20c	Putative transcriptional regulator
AfsS	Global regulator of antibiotic production
SCD6.03c	Global regulator of antibiotic production
SCF55.32	Putative transcriptional regulatory protein
2SCD46.41c	Hypothetical protein
2SCK36.13	Conserved hypothetical protein
SC9A10.09	Hypothetical protein
SCE9.18c	Possible Membrane Protein
2SC7G11.22c	Secreted protein
2SC7G11.18c	Hypothetical protein
SC2A11.12	Oligopeptide transport system integral membrane protein
SC2A11.14	Oligopeptide ABC transporter ATP-binding
SC2A11.11	Putative oligopeptide-binding lipoprotein
SC2A11.10	Oligopeptide transport integral membrane protein

fold at 18 hours, and about 3-4.5 fold at 36 hours. SCF55.32, like *afsS*, was previously found to be induced by BldG overexpression, and this gene is known to be co-transcribed with an upstream gene (SCF55.31) encoding a putative lipoprotein (K. Colvin, unpublished). SCI30A.20c was found to be repressed by ORF3 overexpression (see Table 3.1), and it is located downstream of SCI30A.21c, a gene that was demonstrated to form an operon with SCI30A.20c (K. Colvin, unpublished), and which was induced by BldG overexpression (Table 3.1). Thus *afsS*, SCF55.32, SCF55.31, SCI30A.20c, SCI30A.21c and SCD84.08c all appeared to be good candidates for genes whose transcription was possibly regulated by the putative BldG/ORF3-regulated sigma factor. Confirmation of the BldG-dependence on *afsS* and SCF55.31-.32 transcription has been done using northern analysis and S1 nuclease mapping (K. Colvin, unpublished), and in both cases, the transcription pattern was similar to that observed by DNA microarray analysis. These experiments also revealed that transcription of *afsS* and of SCF55.31-.32 is significantly reduced in the *bldG* mutant strain compared to the wild type strain, and work is currently being carried out in order to confirm the BldG-dependence of SCI30A.20c, SCI30A.21c and SCD84.08c transcription.

As previously mentioned, the transcription of *afsS* is dependent upon the AfsR transcriptional regulator (Lee *et al.*, 2002). AfsR itself is phosphorylated by the AfsK, SCD10.09 and PkaG Ser/Thr kinases (Umeyama *et al.*, 2002), and phosphorylation has been shown to enhance the binding of AfsR to the *afsS* promoter (Lee *et al.*, 2002). In addition, it has been shown that the kinase activity of AfsK is regulated by the KbpA protein, which directly binds to AfsK and inhibits autophosphorylation (Umeyama & Horinouchi, 2001). The BldG-dependence of *afsS* transcription, therefore, may operate

through the regulation of a sigma factor that is responsible for the transcription of *afsS*, *afsK*, *afsL*, *pkaG* or *afsR*, directly, or that is responsible for the transcription of an unknown regulatory protein that is required to induce transcription of *afsK*, *afsL*, *pkaG* and/or *afsR*, or to repress transcription of *kbpA*. Analysis of the microarray data for both the BldG overexpression experiment and the wild type versus *bldGC103* experiment, however, indicated that transcription of *afsR*, SCD10.09 and *pkaG* is not affected by BldG, whereas there was insufficient data to draw any conclusions about the effect of BldG on *afsK* and *kbpA*.

Another set of genes which were affected by *bldG* included SC2A11.10, SC2A11.11, SC2A11.12, SC2A11.13 and SC2A11.14, which are all possibly transcribed as a polycistronic transcript, and which encode components of an ABC oligopeptide transport system. Results of the microarray experiments indicated that these genes were all repressed in the wild type strain ~3-5 fold at 24 hours and ~5-10 fold at 48 hours, while expression levels were similar at 18 and 36 hours, in comparison to the 15 hour time point (Figure 3.8.6). In the mutant strain, however, expression was the same as the 15 hour time point at 18 and 24 hours, and was slightly induced in the later time points. The 2SCK36.13 gene, encoding a conserved hypothetical protein, was found to be repressed at all time points examined in J1501 compared to 15 hours incubation, while expression levels were similar to the 15 hour sample at all time points in the mutant strain. This gene was also previously shown to be induced in response to BldG overexpression (and possibly repressed in response to ORF3 overexpression, see Table 3.1), and therefore is possibly a true indirect target of ORF3 and/or BldG.

Chapter 4:
Discussion

4. Discussion

The *bld* genes of *Streptomyces coelicolor* are known to be involved in the regulation of antibiotic production and/or morphological differentiation, since *bld* mutants are defective in the formation of aerial hyphae, and many are also unable to synthesize one or more of the four antibiotics produced by this organism. This thesis has dealt with the characterization of one of these genes, *bldG*, as well as a downstream gene, *orf3*, in order to determine how *bldG* functions to control morphological and physiological differentiation in *S. coelicolor*, and whether *orf3* is also involved in this process.

4.1 Organization and Expression of the *bldG* Locus

Sequence analysis of *bldG* indicated that it encoded a 113 amino acid protein showing similarity to the SpoIIAA and RsbV anti-anti-sigma factor proteins of *Bacillus* spp. as well as to putative anti-anti-sigma factors found in other organisms like *Staphylococcus* spp., *Listeria* spp. and *Mycobacterium* spp. In *Bacillus*, these proteins function along with cognate anti-sigma factors to regulate the activity of alternative sigma factors that are involved in sporulation and in response to a variety of physiological and environmental stresses. An open reading frame located downstream of *bldG* and designated *orf3* was found to encode a 143 amino acid protein that is very similar to anti-sigma factor proteins found in *Bacillus* spp. and in other organisms. Since genes encoding anti-sigma factors and anti-anti-sigma factors that function together to regulate a target sigma factor are often found as part of an operon, the *orf3* protein

product was regarded as a potential regulatory partner for BldG. Although the target sigma factor is also typically part of the same operon, a sigma factor gene was not found in the vicinity of the *bldG* locus. So far, there is only one other reported example of genes encoding anti-anti-sigma factors and anti-sigma factors that are found at a distant location from the gene encoding the sigma factor. In *Synechocystis* sp. Strain PCC 6803, the *icfG* gene cluster encodes an anti-sigma factor, two anti-anti-sigma factors, and a protein phosphatase, however the putative target sigma factor gene is located elsewhere on the chromosome (Shi *et al.*, 1999). Analysis of the annotated *S. coelicolor* genome sequence revealed that there are at least two other cases of this alternative genetic organization in this organism, and there are also several examples of putative anti-anti-sigma factor genes that are found next to neither sigma factor nor anti-sigma factor genes. Although functional studies have not been performed, if these gene products do in-fact function like their homologs in other organisms, then it might be concluded that the physical separation of sigma factor genes from their cognate regulatory genes, as well as the separation of cognate anti-anti sigma and anti-sigma factor genes, is more common in *S. coelicolor* than in other organisms that harbour these genes. The reason for this is unclear, however given the extraordinary number of sigma factor genes that have been found in the genome (the final annotated sequence database lists 65), it is possible that BldG and ORF3-like proteins, if they do in-fact function as sigma factor regulators, are involved in the post-translational control of multiple sigma factors as opposed to just one sigma factor.

In addition to the absence of an operon-encoded sigma factor, the *bldG* locus differs from other operons such as the *B. subtilis* *spoIIA* and *rsb* operons (encoding the

SpolIA and *Rsb* regulatory proteins, respectively) in the way that expression of the genes is controlled. In the case of both the *spolIA* and the *rsb* operons, the anti-anti-sigma factor, the anti-sigma factor and the sigma factor are expressed as a single transcript from either a σ^H -dependent promoter in the case of *spolIA* (Wu *et al.*, 1991), or a σ^A -dependent promoter in the case of *rsb* (Wise & Price, 1995). Expression of the genes is also upregulated from a second promoter that is controlled by the operon-encoded sigma factor (Schuch & Piggot, 1994; Wise & Price, 1995). Transcriptional analysis of the *bldG* locus, on the other hand, revealed a complex transcription pattern involving not only two different promoters, but also the production of two differently sized *bldG* transcripts. The longer and less abundant of the two transcripts includes both the *bldG* and *orf3* coding regions, while the shorter, more abundant transcript terminates after the second of two hairpin structures that are present in the *bldG-orf3* intergenic region. This *bldG* monocistronic transcript could conceivably arise as a result of endo- or exonuclease cleavage of the *bldG-orf3* polycistronic transcript, in which case the intergenic hairpins might serve to stabilize the remaining *bldG*-containing region and prevent the further degradation of the transcript. Alternatively, the shorter mRNA transcript may result from transcription termination in the intergenic region, where the second hairpin structure might function as a rho-independent transcription termination signal. Although a string of U residues is not present downstream of this inverted repeat, it is well documented that inverted repeats without poly-U tails can act as terminators in *Streptomyces* (Ingham *et al.*, 1995). If the hairpin structure does in-fact function as a transcription terminator, then a mechanism must exist in order to allow readthrough of this terminator to generate the *bldG-orf3* polycistronic transcript. It has been speculated

based on recent studies in our lab that a putative RNA helicase that is divergently transcribed from *bldG* might function in an antitermination mechanism to allow transcription through the intergenic region (Stoeckl, 2001; K. Gislason, unpublished). In this case, the RNA helicase may be required to unwind the hairpin structures in the intergenic region of the mRNA, thereby allowing the RNA polymerase to continue to transcribe the *orf3* gene. An intriguing possibility raised by comparison of transcript abundance observed by northern analysis, primer extension and 5' S1 nuclease mapping (see Sections 3.3.1, 3.3.4 and 3.3.5), is that transcription from the more distal *bldG* promoter (P2) may give rise to the longer *bldG* transcript, whereas expression from the more proximal *bldG* promoter (P1) may give rise to the shorter *bldG* transcript. This implies that the efficiency of transcription termination in the intergenic region is somehow dependent on the promoter unit from which transcription was initiated. That some prokaryotic promoter units allow substantial readthrough of strong terminator signals has been documented (Goliger *et al.*, 1989; Telesnitsky & Chamberlin, 1989), although the exact mechanism by which this might occur is not known.

Recently in *E. coli*, it has been demonstrated that the *clpP-clpX* operon encoding the peptide-degrading component and the ATP-binding component of the Clp protease, respectively, is also transcribed both as a *clpP-clpX* polycistronic transcript and a *clpP* monocistronic transcript (Li *et al.*, 2000). The shorter transcript in this case is thought to arise as a result of transcription termination in the *clpP-clpX* intergenic region, and production of this transcript was also shown to be dependent on the heat shock sigma factor σ^H . Since a σ^H -dependent promoter was found upstream of *clpP* along with two σ^{70} -dependent promoters (Gross, 1996), and an increase in transcription from the σ^H -

dependent promoter correlated with an increase in production of the shorter transcript during carbon deprivation (Li *et al.*, 2000), it was suggested that transcription from the σ^H -dependent promoter resulted in production of the shorter transcript. However exposure to heat, which activates σ^H , did not significantly increase the levels of the shorter transcript, which indicates that expression from the σ^H -dependent promoter alone is not sufficient to increase the production of the shorter transcript. Thus while it seems possible that the generation of the two *bldG* transcripts is dependent on the promoter from which transcription initiated, it is clear that further investigation into this is necessary.

Transcription from the two *bldG* promoters was shown to be independent of *bldG* itself, which suggests that unlike the situation with the *spolIA* and *rsb* operons, the sigma factor proposed to be regulated by BldG is not involved in expression from either promoter. Expression of both the *bldG* monocistronic and the *bldG-orf3* polycistronic transcript, as well as production of BldG and ORF3 protein, was determined to be upregulated at the time when aerial hyphae and pigmented antibiotics are both visible in *S. coelicolor* surface-grown cultures. Interestingly, the amount of BldG protein present greatly exceeded the ORF3 protein levels throughout growth, which correlated with the transcription profile detected by northern analysis. This observed excess of BldG over ORF3 production is in contrast to the situation in *B. subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes*, where it is known or predicted that equimolar concentrations of the cognate regulatory proteins encoded by the *spolIA* and/or *rsb* operons are produced as a result of translational coupling of the genes (Becker *et al.*, 1998; Kalman *et al.*, 1990; Magnin *et al.*, 1997). Possibly, the excess BldG protein may allow the anti-anti-sigma

factor to interact with other anti-sigma factors besides ORF3 in order to regulate multiple sigma factors. Alternatively, it may ensure that activation of the sigma factor takes place under the appropriate conditions. The disappearance of the ORF3 protein in the later stages of development when *bldG-orf3* polycistronic transcript was still readily detected suggests that the ORF3 protein may be subjected to proteolysis, presumably when it is no longer needed for its function. Similar observations have been made for the RseA anti-sigma factor of *E. coli* which regulates the activity of σ^E , an ECF sigma factor that transcribes genes in response to extracytoplasmic stress (Mecsas *et al.*, 1993). RseA, which is located in the cell membrane, is degraded in response to the accumulation of misfolded proteins in the periplasm that occurs during stress, which then leads to release of σ^E (Ades *et al.*, 2003; Ades *et al.*, 1999; Kanehara *et al.*, 2002). Likewise the CarR anti-sigma factor of *Myxococcus xanthus*, which regulates the CarQ ECF-sigma factor involved in the synthesis of carotenoids, is degraded in response to light, which results in disruption of the CarR-CarQ complex (Browning *et al.*, 2003). In addition, the SpoIIAB anti-sigma factor of *B. subtilis* has recently been shown to be subjected to proteolysis, which may ensure that σ^F becomes activated in the forespore compartment (Pan *et al.*, 2001). Mutational analysis indicated that residues at the C-terminus are important for SpoIIAB degradation, however similar residues could not be identified in the ORF3 C-terminal region, which suggests that the mechanism of ORF3 proteolysis may be different than that observed for SpoIIAB.

4.2 The Involvement of *bldG* and *orf3* in the Regulation of Morphological and Physiological Differentiation in *S. coelicolor*

The designation of *bldG* as a regulator of aerial hyphae formation and antibiotic production was originally based on the observed mutant phenotype of the *bldGC103* point mutant strain. To confirm that *bldG* is in-fact involved in these developmental processes, a deletion mutant was constructed to ensure that no BldG protein would be present in the organism, and it was designed as an in-frame deletion in order to avoid any polar effects on the downstream *orf3* gene. The resulting mutant, $\Delta bldG$ 1DB, had the same phenotypic appearance as the *bldGC103* strain, and introduction of wild type *bldG* alone into $\Delta bldG$ 1DB was able to restore aerial hyphae and antibiotic production, suggesting that the deletion of *bldG* was causing the observed mutant phenotype.

Sequence analysis of *bldG* from the *bldGC536* point mutant strain obtained from Wendy Champness revealed that it contained an A $\rightarrow$ T transition mutation which generated a truncated 87 amino acid protein. The *bldGC107* mutant (also from Wendy Champness), on the other hand, contained a base insertion which caused a frameshift mutation that altered the amino acid sequence of the protein starting at position 56, and truncated the protein at position 73. Since both these mutant proteins, as well as the mutant BldG protein from *bldGC103* (which has the identical mutation as *bldGC536*), could not be detected in crude extracts using anti-MBP-BldG antibodies, this suggests that the C-terminal region of BldG may be important for protein stability, although the mere fact that the proteins were truncated might also have targeted them for proteolysis.

The involvement of *orf3* in the regulation of development was suggested by the fact that it encodes a protein that could potentially interact with the BldG anti-anti-sigma

factor protein, and by the fact that *orf3* was shown to be co-transcribed along with *bldG* (see Sections 3.3.2 and 3.3.3). Since point mutations in this gene have never been isolated, however, it was not known what the phenotypic effects of an *orf3* mutation would be. Initial attempts to construct an *orf3* mutant strain involved introducing an antibiotic resistance cassette into *orf3*, and constructing an *orf3* in-frame deletion. Neither of these methods was successful, however, which suggested that either the strategies chosen were not very efficient, or that the removal of *orf3* might be lethal in *S. coelicolor*. A PCR targeting-based gene disruption protocol was then attempted, since it had been reported that this method was rapid and easy to perform, and was more efficient at generating mutant knockouts in *S. coelicolor* than previous classical methods. The result was the isolation of nine *orf3* deletion mutant strains that displayed a variety of phenotypes with regards to aerial hyphae formation and antibiotic production, many of which were distinct from that of the wild type strain depending on the growth medium used. Interestingly, one of the mutant strains, 2-8aiii, was unable to grow on a medium (ISP-4) composed of inorganic salts and starch as a sole carbon source, suggesting that the mutation in this strain may have resulted in a defect in starch metabolism. Alternatively, the deletion of *orf3* may have resulted in the inability of the strain to synthesize one or more amino acids, since both R2YE agar and MS agar contain amino acids while ISP-4, a defined minimal medium, lacks any amino acids. Introduction of the wild type *orf3* gene on an integrative plasmid was unable to restore the wild type phenotype to any of the mutant strains, which implied that the mutants contained secondary site mutations in one or more genes. However in the case of the 2-8d and the 2-8ai strains, production of ORF3 was not observed when the strains were complemented

with pAU324 (pSET Ω +*orf3*), which might explain the inability of pAU324 to restore the wild type phenotype to these two strains. The idea that the deletion of *orf3* could cause secondary site mutations is supported by studies of the *B. subtilis* *spoIIAB* anti-sigma factor and the *S. coelicolor* RsrA and the RsuA anti-sigma factors, where disruption or deletion of the anti-sigma factor gene resulted in the acquisition of secondary site mutations in the corresponding sigma factors (Gehring *et al.*, 2001; Paget *et al.*, 2001b; Schmidt *et al.*, 1990). It is thought that these suppressor mutations serve to reduce the activity of the target sigma factor in order to avoid any toxic effects under conditions where it is normally inactivated. In the case of the *orf3* deletion mutants, the presence of active sigma factor(s) during a stage in development when it would normally be repressed by ORF3 may disrupt the intricate regulatory network that controls development, and the acquisition of mutations in the sigma factor(s) may help to reduce the lethal effects of such a disruption. Whether the proposed secondary site mutations actually occur within the sigma factor gene(s), however, has not yet been determined, since the identity of the target sigma factor(s) is unknown. The existence of such secondary site mutations makes it difficult to determine the exact phenotypic effect of an *orf3* deletion mutation. To solve this problem, one could attempt to delete *orf3* in a strain in which a second copy of *orf3* under control of an inducible promoter is present on an integrative plasmid elsewhere in the chromosome. Deletion of the wild type *orf3* gene could then be carried out under conditions where the second copy is expressed, and the phenotypic effects of the deletion could be assessed by simply shutting off expression of the plasmid copy of *orf3*.

4.3 Regulation of BldG Activity in *S. coelicolor*

The SpoIIAA and RsbV anti-anti-sigma factors of *B. subtilis* are known to be regulated by phosphorylation, since the phosphorylated forms of these proteins are unable to reverse the anti-sigma factor-mediated inhibition of the target sigma factors. More recently, the Slr1865 putative anti-anti-sigma factor of *Synechocystis* sp. Strain PCC 6803 has also been shown to be phosphorylated (Shi *et al.*, 1999), and phosphorylation is thought to be involved in the regulation of the RsfB anti-anti-sigma factor, which controls the activity of the *M. tuberculosis* stress response sigma factor σ^F (Beaucher *et al.*, 2002). In all of these cases, the phosphorylation site is a single serine residue (Beaucher *et al.*, 2002; Najafi *et al.*, 1995; Shi *et al.*, 1999; Yang *et al.*, 1996) which is highly conserved in a number of known or putative anti-anti-sigma factors (see Figure 3.1.1), suggesting that the reversible phosphorylation of anti-anti-sigma factors may be a widespread mechanism used to regulate these proteins. The only exception to this which has been documented is the RsfA anti-anti-sigma factor of *M. tuberculosis*, which also functions to regulate the σ^F stress-response sigma factor in that organism (Beaucher *et al.*, 2002). Sequence analysis revealed that RsfA contains a cysteine residue in the position corresponding to the phosphorylated serine residue of SpoIIAA, and a computer model of the protein showed that this residue could potentially interact with a second cysteine residue to form a disulfide bond. In addition, RsfA could only bind to its cognate anti-sigma factor under reducing conditions, and mutation of either cysteine residue resulted in constitutive

binding even under oxidizing conditions, which suggests that RsfA is regulated by the redox potential of the cell (Beaucher *et al.*, 2002).

Protein sequence analysis revealed that as with most other putative anti-anti-sigma factors, the serine residue that is phosphorylated in SpoIIAA is conserved in BldG, suggesting that BldG may also be regulated by phosphorylation. That BldG is phosphorylated was confirmed using *in vitro* phosphorylation assays which demonstrated that a MBP-BldG fusion protein could be phosphorylated by a kinase present in *S. coelicolor* crude extracts, and that ATP serves as the phosphoryl group donor. This BldG-specific kinase appears to be partially dependent on BldG itself, since phosphorylation of MBP-BldG was significantly reduced when extract from the $\Delta bldG$ 1DB or the *bldGC107* mutant strains was used, and a high level of MBP-BldG phosphorylation could be restored by the presence of wild type BldG in the $\Delta bldG$ 1DB extract. Phosphorylation of BldG was also shown to be abolished *in vitro* and *in vivo* when Ser-57 was changed to an alanine, suggesting that this residue serves as the sole phosphorylation site in BldG. While it could be argued that the S→A mutation at this position disrupts the protein structure such that it cannot interact with the kinase in order to be phosphorylated, it has been demonstrated in *B. subtilis* and *M. tuberculosis* that mutating the phosphorylatable serine residue to an alanine does not affect the overall structure of SpoIIAA or RsfB, since the proteins were still able to reverse the anti-sigma factor-mediated inhibition of the corresponding target sigma factors (Beaucher *et al.*, 2002; Diederich *et al.*, 1994). As well, recent studies of the structure of the SpoIIAA protein from *B. sphaericus* demonstrated that the phosphorylatable serine residue in that protein is oriented away from the protein body, and does not form any polar interactions

with other amino acids (Seavers *et al.*, 2001). Since structural modeling of the BldG protein using SWISS-MODEL had predicted a similar orientation of the phosphorylatable serine residue (see Section 3.1.2), this suggests that the S→A mutation would not significantly affect the BldG protein structure.

While the exact effect that phosphorylation has on BldG function has yet to be determined, if BldG functions in a manner analogous to homologous proteins in *Bacillus* and in other organisms, then phosphorylation might convert BldG into a form that is no longer able to interact with its binding partner. Studies into the phosphorylation of SpoIIAA had initially suggested that phosphorylation altered the conformation of the protein such that it was no longer able to interact with its binding partner (Magnin *et al.*, 1996). More recently, however, it was demonstrated that the structural changes of SpoIIAA induced by phosphorylation are slight, and that the mere presence or absence of the phosphate group probably determines whether SpoIIAA will bind to SpoIIAB or not (Seavers *et al.*, 2001). The fact that protein extract from the $\Delta bldG$ 1DB null mutant strain expressing BldGS57A was able to phosphorylate MBP-BldG whereas phosphorylation by the null mutant extract alone was barely detectable, suggests that the unphosphorylated form of BldG is likely the active form of the protein. Interestingly, the *bldGS57A* mutant allele showed a similar phenotype as the *bldG* null mutant, when it was expected that the two mutations would exhibit opposing phenotypic effects. Similar results have been observed in *B. subtilis* with SpoIIAA, however (Diederich *et al.*, 1994; Duncan *et al.*, 1996), which suggests that the regulation of anti-anti-sigma factor activity is necessary for proper development in both organisms.

In *Bacillus*, phosphorylation of the anti-anti-sigma factor is carried out by the cognate anti-sigma factor itself. These anti-sigma factor proteins show similarity to a family of histidine protein kinases involved in bacterial two-component signal transduction systems (Duncan & Losick, 1993; Kang *et al.*, 1996; Min *et al.*, 1993). Unlike histidine kinases, however, they lack the conserved histidine residue, the autophosphorylation activity, and the phosphatase activity, and they phosphorylate their target protein on a serine residue rather than an aspartate residue (Dutta *et al.*, 1999; Stock *et al.*, 1995). The ORF3 putative anti-sigma factor was the first obvious choice for a kinase that could phosphorylate BldG, however this does not appear to be the case for a number of reasons. First, alignment of the SpoIIAB and RsbW protein sequences with that of ORF3 indicated that ORF3 possessed only two of the five signature residues of the N, D and G boxes that are present in histidine kinases and in the *Bacillus* proteins. The D box, which contains a DXG motif, is thought to be involved in the coordination of Mg^{2+} , while the G-box, which is a glycine-rich region, is involved in ATP binding, and the N-box may function in protein-protein interactions (Garsin *et al.*, 1998; Stock *et al.*, 1995). Also, my analysis of the ORF3 protein sequence using the Prosite ProfileScan Server, and analysis by another researcher using the Pfam and SMART databases (Mittenhuber, 2002), did not reveal the presence of any kinase domains in the protein. In addition, it was demonstrated that phosphorylation of MBP-BldG was barely detectable when the $\Delta bldG$ 1DB crude extract was used, which was shown by western analysis to contain ORF3 protein. It therefore seems likely that another protein that possesses serine kinase activity is responsible for the phosphorylation of BldG. A similar situation has been observed in *Synechocystis* sp. Strain PCC 6803, where the Slr1859 anti-anti-sigma factor

was found to be phosphorylated with very low efficiency by the neighbouring anti-sigma factor Slr1861 (Shi *et al.*, 1999). As well, the RsfB anti-anti-sigma factor of *M. tuberculosis* was shown to interact with the UsfX anti-sigma factor, however UsfX was unable phosphorylate RsfB (Beaucher *et al.*, 2002). A PSI-BLAST search of the *S. coelicolor* genome (http://www.sanger.ac.uk/Projects/S_coelicolor/) has revealed the presence of at least 47 RsbW-related proteins, many of which (37) are predicted to possess kinase activity (Mittenhuber, 2002). Interestingly, at least 16 of these proteins are also predicted to function as protein phosphatases. Possibly, one or more of these proteins is responsible for the reversible phosphorylation of BldG.

4.4 Analysis of BldG-ORF3 Protein Interactions

The proximity on the chromosome of the genes encoding BldG and ORF3, together with the transcript analysis showing that they are expressed as an operon, led to the hypothesis that BldG might work together with ORF3 to regulate a target protein such as a sigma factor. Two different protein binding experiments were therefore performed in order to investigate BldG-ORF3 protein interactions. The first experiment, affinity chromatography, looked at whether purified His₁₀-ORF3 or ORF3 overexpressed in *S. coelicolor* crude extract could be co-purified with MBP-BldG that was bound to amylose resin. However, ORF3 (and His₁₀-ORF3) did not appear to be able to bind to MBP-BldG under the conditions used, and the addition of ADP, which has been shown to influence the formation of the SpoIIAA-SpoIIAB complex, had no effect. While it is possible that the buffer used in the assay was inappropriate for promoting complex formation, a similar experiment performed using a buffer previously shown to promote complex formation

between a GST-tagged protein and various test proteins (Allen *et al.*, 2002), also failed to show interaction between BldG and ORF3 (B. Leskiw, personal communication). Furthermore, chemical cross-linking experiments were unable to demonstrate binding between BldG and ORF3, and in this case untagged BldG and ORF3 proteins were used in order to avoid any complications arising from the presence of a tag on either protein. A ~35 kDa BldG-containing complex as well as other BldG-containing complexes (>85 kDa) were detected in the cross-linking experiment, and although this experiment has not yet been done, purification of these complexes using anti-MBP-BldG antibodies will allow for the identification of the interacting protein(s) using mass spectrometry. Once the nature of the other protein(s) is known, protein binding will have to be verified by other methods, since it is possible for cross-linking between neighbouring proteins to occur even though they are not in direct contact with one another (Phizicky and Fields, 1995).

The question then arises: do BldG and ORF3 interact to form a complex in *S. coelicolor*? The fact that negative results were observed for both affinity chromatography and chemical cross-linking does not necessarily mean that the two proteins do not interact *in vivo*. For example, it is conceivable that the interaction between BldG and ORF3 may be sufficiently weak that complex formation cannot be detected by affinity chromatography. Similar findings have been demonstrated using a GST-tagged protein, where weak interactions ($K_D > 2\mu\text{M}$) could not be detected (Allen *et al.*, 2002) since they most likely could not survive the extensive washings of the immobilized proteins. As well, it is possible that MBP-BldG is not able to interact both with ORF3 and with the amylose resin due to steric interference by the MBP tag. In the

case of the chemical cross-linking experiments, the cross-linker used, BS3, reacts with the ϵ -amino group of lysine residues that are about 11 Angstroms apart [(Mattson *et al.*, 1993); Pierce catalog]. If, however, there are no lysine residues on BldG or ORF3 with accessible ϵ -amino groups that are properly positioned to allow cross-linking to occur, then a BldG-ORF3 complex will not be isolated after SDS-PAGE. In order to demonstrate conclusively that complex formation does not occur using chemical cross-linking, a variety of cross-linkers with spacers of different length and geometry should be tested. For example, the EGS cross-linker, like BS3, reacts with ϵ -amine group of lysine residues, however the spacer length is 16 Angstroms as opposed to 11.4 (Alper *et al.*, 1994). In addition, cross-linkers that react with other groups besides primary amines such as sulfhydryls, carbonyls, and carboxylic acids could also be tested, as well as cross-linkers that react with different targeted functional groups on each protein (i.e. heterobifunctional cross-linkers).

In addition to affinity chromatography and chemical cross-linking, a number of other methods are available which could be used for detecting protein-protein interactions. For example, co-immunoprecipitation experiments could be performed where *S. coelicolor* crude extracts are incubated with anti-MBP-BldG antibodies that are bound to protein A-Sepharose, and any proteins that interact with BldG *in vivo* would be precipitated along with BldG. If the interaction is weak as suggested above, chemical cross-linkers could be used prior to immunoprecipitation in order to stabilize the complex. Preliminary experiments have indicated that the anti-MBP-BldG antibodies are effective in immunoprecipitating BldG from *S. coelicolor* crude extracts (not shown; B. Leskiw, personal communication), which suggests that this might serve as a reasonable

method for determining whether ORF3 (or any other proteins) interacts with BldG *in vivo*. Also possible is the use of the two-hybrid system, which involves creating a fusion of one protein (X) to the DNA binding domain of a transcriptional activator, and of another protein (Y) to the transcriptional activation domain of the activator protein (Phizicky & Fields, 1995). The two fusion proteins are expressed in the same cell, and if proteins X and Y are able to interact, then the DNA binding domain and the transcription activation domain come together, which then allows for expression of one or more reporter genes. The main advantage of this method is that it is highly sensitive, detecting interactions that are not detected by other methods. For example, the two-hybrid system was used to detect complexes of the eukaryotic RAS proteins with certain protein kinases that could not be detected by either co-immunoprecipitation or chemical cross-linking (Van Aelst *et al.*, 1993). It is therefore possible that this method might allow detection of BldG-ORF3 interactions.

4.5 Identification of Indirect Targets of BldG and ORF3

Since a sigma factor gene is not present within the *bldG* locus, it was not known which of the 65 sigma factors present in *S. coelicolor* would represent the putative target(s) of BldG and ORF3. DNA microarray analysis was therefore employed to identify indirect targets of BldG and ORF3 in hopes of using this information to pinpoint the sigma factor(s) that may be regulated by the two proteins. Three different experiments were performed, starting with one in which gene expression was monitored in response to overexpression of ORF3 in wild type *S. coelicolor*, and one in which gene expression was monitored in response to BldG overexpression in the *bldGC103* mutant

strain. The idea behind these experiments was that if ORF3 binds a sigma factor and prevents its association with core RNA polymerase, and BldG counteracts this inhibition by forming a complex with ORF3, then the overexpression of each protein would have opposing effects on the activity of the sigma factor, and therefore on transcription of genes under control of this sigma factor.

Unfortunately, very few genes that displayed this opposing transcription pattern were detected in the two experiments. This is partly explained by the fact that many of the genes that were repressed or induced in response to ORF3 overexpression were not represented in the data collected for the BldG overexpression experiment, and vice versa. The absence of data for a particular gene would typically occur when the spot on the array was bad due to either flaws in the array itself, from dust or debris on the array that occluded the spot, or from high non-specific background that prevented an accurate reading (B. Leskiw, personal communication).

For those genes affected by ORF3 or BldG overexpression that were represented in the data, the lack of opposing transcription patterns could be due to a couple of reasons. First, the growth medium used in the ORF3 overexpression experiment was different from that used in the BldG overexpression experiment. In the first case, the defined minimal medium NMMP containing glucose as the carbon source was used, whereas R2YE which is a rich, undefined medium, was used in the BldG overexpression experiment. The reason for this difference in growth medium was that the *bldGC103* mutant strain was unable to grow sufficiently in NMMP medium. Secondly, the OD at which protein overexpression was induced in the two strains was about 0.5, which roughly corresponds to early-mid log phase (although growth curves would need to be

performed to verify this). If BldG and ORF3 regulate the same sigma factor, then it is predicted that the effect of the two proteins on sigma factor activity will be exhibited at different stages of growth. For example, BldG may bind to ORF3 early during growth, allowing sigma factor-directed transcription to occur. If BldG is overexpressed during this stage, there will be very little effect on sigma factor activity, since the sigma factor will already be active. On the other hand, overexpression of ORF3 will result in a significant decrease in sigma factor activity, since the excess ORF3 present can sequester the sigma factor away from RNA polymerase. Conversely at another stage of growth, BldG may be inactive towards ORF3, allowing ORF3 to bind the sigma factor and inhibit its activity. Overexpression of BldG at this stage, therefore, would result in release of the sigma factor, whereas ORF3 overexpression would have no effect on sigma factor activity. Consequently the stage of growth at which protein overexpression is induced may be critical to the identification of genes whose transcription is affected by both BldG and ORF, and experiments should be performed such that each protein is induced at early and late stages of growth in similar growth media.

Despite all of this, a number of putative BldG and ORF3 indirect targets were identified by overexpression of BldG and ORF3, as well as by performing a third experiment in which the transcription pattern of genes in the wild type strain and in the *bldGC103* mutant strain were compared during the course of development. Two of these identified genes, *afsS* and SCF55.32 (which is co-transcribed with SCF55.31), have been verified to be BldG targets by northern analysis and/or S1 nuclease mapping (K. Colvin, unpublished), and *afsS*, SCF55.32, SCD84.08c, and SCI30A.20c all showed the same transcription pattern in the wild type and the *bldG* mutant strain, suggesting that

SCD84.08c and SCI30A.20c are most likely true BldG targets as well. In addition, DNA microarray analysis indicated that 2SCK36.13 and SCI30A.20c were both induced in response to BldG overexpression, and *afsS*, 2SCK36.13 and SCI30A.21c (which is co-transcribed with SCI30A.20c) were likely repressed in response to ORF3 overexpression. Thus *afsS*, SCF55.31-.32, SCI30A.20c-.21c, and SCD84.08c may all represent direct or indirect targets of a BldG/ORF3-regulated sigma factor, although verification of this by other methods such as S1 nuclease mapping will need to be performed.

afsS encodes a global regulator of antibiotic production, and S1 nuclease mapping experiments indicated that transcription of *afsS* is significantly reduced in the *bldGC103* mutant, which could partially account for the defect in antibiotic production observed in the *bldG* mutant strain. Expression of *afsS* is known to be dependent on the AfsR transcriptional regulator, and phosphorylation of AfsR by the AfsK, SCD10.09 and PkaG Ser/Thr kinases has been shown to enhance the binding of AfsR to the *afsS* promoter (Lee *et al.*, 2002; Umeyama *et al.*, 2002). However since expression of *afsR*, SCD10.09 and *pkaG* was not affected in the *bldG* mutant, this suggests that the BldG-dependence of *afsS* expression is not manifested through these regulatory genes, and it is unknown whether *bldG* affects transcription of *afsK*. In terms of genes that are likely to be involved in morphological differentiation, only one (STH24.08) encoding a putative sporulation protein, was identified in the microarray experiment using RNA isolated after ORF3 overexpression. *bldN*, which is a known *bldG* indirect target required for aerial hyphae formation (Bibb *et al.*, 2000), was not represented in the data collected, and this may be true for other *bldG*-dependent genes involved in aerial hyphae formation.

At this time, the involvement of the SCF55.31-.32, SCI30A.20c-.21c, 2SCK36.13, and SCD84.08c putative *bldG/orf3* targets in morphological and physiological differentiation is speculative, based on sequence analysis alone. For example, SCF55.32 encodes a putative regulatory protein that shows similarity to AlgP, a regulatory protein required for the transcription of the *algD* gene involved in the production of the exopolysaccharide alginate by *Pseudomonas aeruginosa* (Konyecsni & Deretic, 1990). AlgP in turn shows similarity to the eukaryotic histone H1 protein in the C-terminal domain, and contains a number of highly organized repeats of the Lys-Pro-Ala-Ala (KPAA) motif and its variants within this region (Deretic & Konyecsni, 1990). While the exact function of AlgP is not known, it has been shown that the AlgR response regulator binds to two specific sites in the FUS region located far upstream of the *algD* mRNA start site, and to a third site within a region proximal to the transcription start site (Mohr *et al.*, 1991; Mohr *et al.*, 1990; Mohr *et al.*, 1992). AlgP may then facilitate bending or looping of the *algD* promoter to enhance interactions between the AlgR proteins bound at the three sites (Deretic & Konyecsni, 1990). Analysis of the SCF55.32 protein sequence revealed a section of the protein that is rich in lysine, proline and alanine residues, similar to the AlgP C-terminal region that is involved in DNA binding, although SCF55.32 does not have the repeat motifs present in AlgP. Perhaps SCF55.32 functions in a similar manner proposed for AlgP to regulate expression of genes involved in development.

The SCI30A.21c protein product is a putative transcriptional regulator of the TetR family of regulatory proteins, and like SCF55.32, it may function in regulating the expression of genes involved in morphological and/or physiological differentiation. The

SC2A11.10-11-12-13-14 gene cluster, a likely *bldG* indirect target, encodes components of an oligopeptide transport system, and recent studies have shown that the SC2A11.11 protein product is induced in response to heat and cold shock (Novotna *et al.*, 2003), which suggests that this transport system may be important for adaptation to these stress conditions.

The SCD84.08c gene encodes a homolog of PstC, a component of the Pst (phosphate-specific transport) systems of *E. coli*, *B. subtilis* and *M. tuberculosis* (Braibant *et al.*, 1996; Rao & Torriani, 1990; Takemaru *et al.*, 1996). In *E. coli*, this system which is comprised of PstC, PstA, PstB and PstS, is involved in the transfer of inorganic phosphate (Pi) into the cell under conditions of low external phosphate levels, as well as in the transmembrane signalling event that controls the phosphate (Pho) regulon (Rao & Torriani, 1990). PstC together with PstA forms the transmembrane channel in the cytoplasmic membrane for phosphate transport, while PstB, which is linked to the channel on the cytoplasmic side of the membrane, is thought to provide the energy needed for Pi transport. PstS is a periplasmic Pi binding protein that captures Pi and transfers it to the PstA-C transport channel. In *S. coelicolor*, genes showing similarity to *pstS*, *pstA* and *pstB* are found together with SCD84.08c (*pstC*) in the same arrangement and orientation observed in *E. coli* and *B. subtilis*, and like in these organisms, the genes are probably transcribed together as an operon. Thus *bldG* may be involved in the expression of this putative Pi transport system, which, if found to be true, would further support the notion that the nutritional status of the organism is involved in the decision to undergo differentiation in *S. coelicolor*.

The involvement of the various putative *bldG/orf3* targets in morphological and/or physiological differentiation will need to be assessed by constructing knockout mutations of the genes and observing the phenotypic effects of such knockouts. Such work is currently underway for the SCI30A.20c-.21c operon as well as the SCF55.31-.32 operon (K. Colvin, unpublished). Currently, the sigma factors that control expression of these various genes are unknown, although alignment of the putative promoter sequences for *bldN*, *afsS*, SCF55.31-.32 and SCI30A.21c-.20c [(Bibb *et al.*, 2000); K. Colvin, unpublished] revealed a significant amount of similarity between the four promoters, suggesting that the genes may all be recognized by the same sigma factor (Figure 4.5.1). Alternatively, the genes may be transcribed by different sigma factors that recognize similar promoter sequences, a situation that has been observed in *B. subtilis* with the σ^F/σ^G sporulation-specific and the σ^W/σ^X ECF-sigma factors, and in *E. coli* with σ^S/σ^{70} (Hengge-Aronis, 1999; Huang *et al.*, 1998; Sun & Setlow, 1991; Sun *et al.*, 1989).

The *S. coelicolor* genome is known to harbour nine genes encoding sigma factors that are related to the stress response sigma factor σ^B of *B. subtilis*, one or more of which are likely candidates for sigma factors that may be regulated by BldG/ORF3. Out of these nine sigma factors, only six have been characterized to some extent. SigF is involved in the later stages of sporulation, and SigB, SigI and SigJ are all induced in response to osmotic shock, suggesting that they function as stress-response sigma factors. In addition, SigB is also required for proper differentiation of *S. coelicolor*, as a *sigB* null mutant was bald when grown on rich medium, and produced large amounts of actinorhodin (Cho *et al.*, 2001). SigH is another stress response sigma factor that is developmentally regulated and is induced in response to heat, osmotic and ethanol shock,

Figure 4.5.1 Alignment of the promoter sequences of known or predicted BldG indirect targets. The predicted -10 and -35 sequences for each gene are indicated, as well as the proposed transcription start sites (+1) and the sizes of the spacer regions between the initiation site and the -10 sequence, and between the -10 and -35 sequences. A promoter consensus sequence for the genes is shown below the alignment, with capital letters indicating conserved bases in all sequences, lower case letters representing conserved bases in three of the four sequences, and (n) indicating non-conserved bases. Also shown is the proposed consensus sequence for SigB-like sigma factors of *S. coelicolor* (Cho *et al.*, 2002). Alignment kindly provided by K. Colvin.

	<u>-35</u>		<u>-10</u>		<u>+1</u>
SCF55.31	GTGCCT	N=14	GACGCT	N=5	C
<i>bldN</i>	GTGCCT	N=12	TATTCT	N=5	GA
<i>afsS</i>	GTTCGT	N=15	CACTGT	N=6	C
SCI30A.21c	GTGGCG	N=16	GACTCT	N=6	CA
Consensus:	GTgcct	N=12-16	nActcT		
SigB-like:	GnnTnn	N=12-16	GGGtAn		
Consensus					

however it does not appear to play a role in differentiation, since a *sigH* null mutant had a wild type appearance (Viollier *et al.*, 2003a). Disruption of the *sigG* gene also had no obvious effects on growth, morphological differentiation or antibiotic production, and expression of the gene did not occur during vegetative growth or differentiation, which suggests that *sigG* may be expressed only under certain conditions (Kormanec *et al.*, 1999).

The fact that SigF is only required for later stages of sporulation and SigG is not expressed during vegetative growth or differentiation suggests that these sigma factors would not be regulated by BldG/ORF3, since the target sigma factor presumably controls the expression of genes involved in aerial hyphae formation and antibiotic production. As well, SigI and SigJ protein production only occurs under conditions of osmotic shock and not under normal growth conditions (Viollier *et al.*, 2003b), implying that these sigma factors are also not targets of BldG/ORF3 under normal growth conditions. SigH, SigI, SigJ and SigB, are all thought to recognize similar promoter sequences, since the *sigHP2* and *sigBP1* promoters recognized by SigH and SigB, respectively, are similar in sequence to each other, and SigH, SigI and SigJ were all shown to transcribe the *B. subtilis* σ^B -specific *Pctc* promoter *in vitro* (Cho *et al.*, 2001; Kormanec *et al.*, 2000; Viollier *et al.*, 2003b). A comparison of the putative consensus promoter sequence for the BldG/ORF3 indirect targets with that recognized by SigB, SigH, SigI and SigJ indicates that the two sequences are quite different, suggesting that the genes are probably not transcribed by any of these sigma factors (Figure 4.5.1). Of course, it cannot be ruled out at this point that *bldN*, *afsS*, SCF55.31-32 and SCI30A.21c-20c are

indirectly dependent on the BldG/ORF3-regulated sigma factor(s). So far, mutational studies have failed to identify a *B. subtilis* σ^B -like sigma factor that is required for both aerial hyphae formation and antibiotic production, although it is possible that at least one of the three remaining σ^B -like sigma factors that are still uncharacterized is required for these differentiation processes. Given the apparent redundancy of SigB, SigI, SigJ and SigH in terms of promoter recognition, however, it is possible that the deletion of multiple sigma factors is necessary in order to observe any effects on differentiation. Such an idea supports the previous suggestion that BldG/ORF3 in-fact may regulate the activity of multiple sigma factors during development.

4.6 Proposed Models for the Role of BldG and ORF3 in *S. coelicolor*

Differentiation

Although it could not be demonstrated by protein binding studies that BldG and ORF3 are able to form a complex, the fact that the *bldG-orf3* genes are co-transcribed, and that transcription of the *afsS*, 2SCK36.13 and the SCI30A.21c-.20c genes appeared to be repressed by ORF3 overexpression and induced by BldG overexpression, suggests that they do in-fact interact with each other to possibly regulate a sigma factor. In order to formulate a model for how this might occur in *S. coelicolor*, the following observations need to be taken into account. First, the BldG protein is present at all time points examined (15-48 hours post-inoculation) in surface-grown cultures, whereas the ORF3 protein is virtually absent from 48 hour samples, which suggests that this protein is no longer required for its function in the later stages of growth when sporulation has begun. Second, both transcript and protein analysis revealed that BldG is produced in excess of

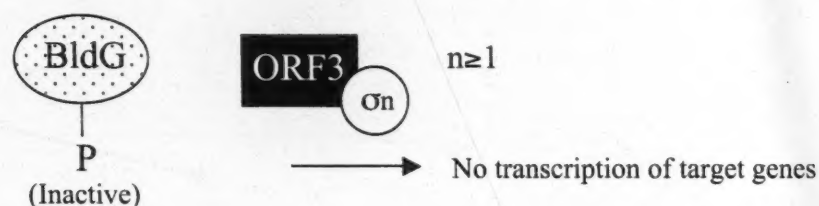
ORF3 protein throughout growth, which implies that BldG may interact with more than one anti-sigma factor during development, or that the excess BldG protein ensures that the target sigma factor becomes active and stays active at the proper stages of growth. Third, unphosphorylated BldG, which likely represents the active form of the protein, is present at much higher amounts than the phosphorylated form (P-BldG) during vegetative growth (15 hours). However, equivalent amounts of the two forms are observed by 24 hours post-inoculation when aerial hyphae and pigmented antibiotics are both visible, and both forms remain at equivalent levels at least until sporulation begins. The increase in amount of P-BldG protein that occurs between vegetative growth and aerial hyphae and pigment production also correlates with the observed increase in activity of the BldG-specific kinase. Finally, the deletion of *orf3* appears to result in the acquisition of secondary site mutations, possibly within the target sigma factor gene(s) itself, suggesting that the target sigma factor(s) is inhibited by ORF3 during the very early stages of vegetative growth, and that the secondary site mutations could have occurred to reduce the toxic effects resulting from the premature release of the sigma factor(s). Since introduction of the BldGS57A mutant protein (which is hypothesized to be constitutively active) into the $\Delta bldG$ 1DB strain would also presumably cause the premature release of the target sigma factor(s) early on in growth, the inability of the BldGS57A mutant protein to complement the bald phenotype of the $\Delta bldG$ 1DB mutant strain may have also been due to the acquisition of secondary site mutations in the target sigma factor gene(s).

In addition to the observations mentioned above, the transcriptional data for *bldN*, a known *bldG* indirect target (although it has not yet been established that *bldN* is also an

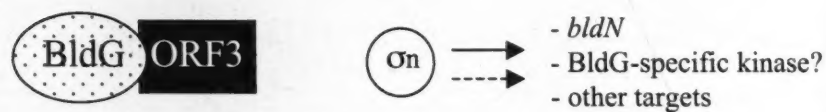
orf3 target), was also taken into account in formulating the proposed models. Whether the models will also apply to other putative *bldG/orf3* targets such as *afsS*, 2SCK36.13, SCF55.31-.32, SCI30A.21c-.20c and SCD84.08c is not known at this point, since additional transcriptional data on these genes will need to be obtained. *bldN* is known to be expressed at very low levels during vegetative growth, and transcription is significantly upregulated when aerial hyphae and pigmented antibiotics are visible (Bibb *et al.*, 2000; Elliot *et al.*, 2001). This temporal pattern of gene expression is dependent on the BldD repressor protein, since transcription was readily detected in RNA samples isolated during vegetative growth from a *bldD* mutant strain (Elliot *et al.*, 2001). On the other hand, *bldN* transcription, and production of BldN protein, was completely abolished throughout development in a *bldH* and a *bldG* mutant strain, which indicates that both of these genes are absolutely required for *bldN* expression (Bibb & Buttner, 2003; Bibb *et al.*, 2000). *bldH* encodes a putative transcriptional activator protein that is predicted to activate transcription of the *bldN* gene directly, since the ortholog of BldH in *S. griseus*, AdpA, has been shown to directly activate transcription of the *bldN* ortholog, AdsA, in the same organism [(Yamazaki *et al.*, 2000); see Section 1.4]. Whether the postulated BldG-regulated sigma factor is directly responsible for the transcription of *bldN*, or whether it functions through transcription of a *bldN* activator protein, is currently not known. It is unlikely, however, that the postulated BldG-targeted sigma factor is directly involved in the transcription of *bldH*, since it has been demonstrated that *bldH* and *bldG* mutants do not fall within the same extracellular complementation group (see Section 1.3).

Figure 4.6.1 Schematic diagram of how BldG and ORF3 might function to control development in *S. coelicolor*. (A) During early vegetative growth (<15 hours post-inoculation), BldG (dotted oval) is likely present exclusively in the inactive, phosphorylated form (P-BldG), which would allow the ORF3 anti-sigma factor (black box) to bind the target sigma factor(s) (white circle) and inhibit sigma factor-directed transcription. (B) By late vegetative growth/transition phase (15 hours post-inoculation), BldG is predominantly in the unphosphorylated, active form, which may then bind ORF3 and allow the sigma factor(s) to direct transcription of genes involved in differentiation. The solid and dotted arrows indicate that the dependence of the target genes on the sigma factor(s) (e.g. *bldN*) may be either direct or indirect. (C) Once aerial hyphae and pigmented antibiotics are both visible (24 hours post-inoculation), BldG is present in both the inactive, phosphorylated form, and the active, unphosphorylated form, the latter of which might still form a complex with ORF3, and allow the sigma factor(s) to continue to transcribe its target genes. Since unphosphorylated BldG is present in the organism at least until sporulation begins (48 hours post-inoculation), and ORF3 is degraded at the onset of sporulation, it is proposed that the sigma factor(s) remain active at least until this stage of development is reached. (n) indicates that more than one sigma factor may be regulated by BldG and ORF3 in this manner.

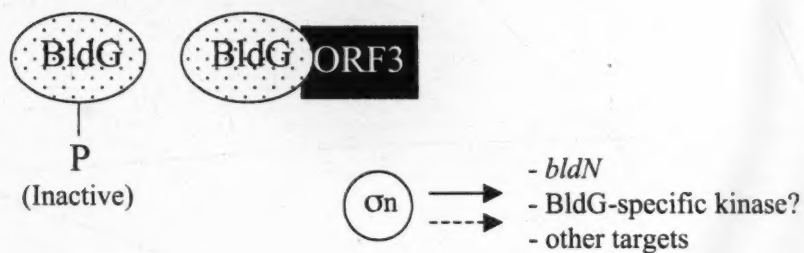
(a) Early vegetative growth (<15 hours post-inoculation):



(b) Late vegetative growth/transition phase
(15 hours post-inoculation):



(c) Onset of aerial hyphae formation and
antibiotic production (24 hours post-inoculation):



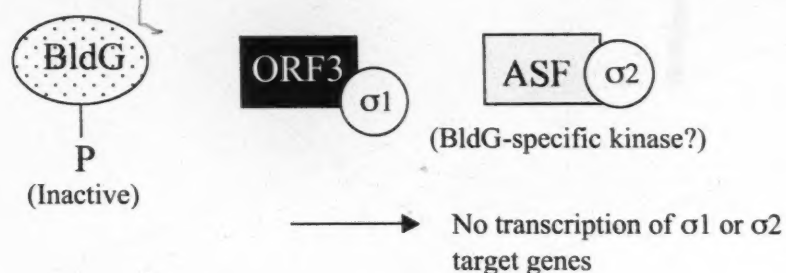
The first model proposing how BldG and ORF3 might function to control development in *S. coelicolor* is shown in Figure 4.6.1. During very early vegetative growth, ORF3 is hypothesized to form a complex with one or more target sigma factors, thereby preventing association of the sigma factor(s) with core RNA polymerase. Since almost all of the BldG protein is present in the active, unphosphorylated form at the earliest time point examined (15 hours), it is proposed that the ORF3-directed inhibition of the target sigma factor(s) occurs at some stage earlier than 15 hours. This is supported by the fact that very small amounts of aerial hyphae can be seen with plate cultures after only 18 hours of incubation, which implies that at 15 hours the cultures are already in the later stages of vegetative growth, and are even possibly into transition phase between primary growth and secondary metabolism and differentiation. During early vegetative growth (<15 hours), therefore, BldG is likely present exclusively in the phosphorylated, inactive form, and by 15 hours post-inoculation, the active form of the protein becomes predominant due to either inefficient phosphorylation by the BldG-specific kinase, or to the activation of a BldG-specific phosphatase enzyme. BldG then binds ORF3, allowing the sigma factor(s) to direct transcription of genes required for differentiation. By the time aerial hyphae and pigmented antibiotics are both visible (24 hours post-inoculation), the level of inactive P-BldG increases such that it is the same as that observed for unphosphorylated BldG. This increase in P-BldG, and the observed increase in BldG-specific kinase activity, might result from increased expression of the gene encoding the BldG-specific kinase by the BldG-regulated sigma factor (either directly or indirectly), since it has been shown that phosphorylation is partially dependent on the presence of active BldG. Alternatively, the activity of the kinase enzyme, as opposed to the amount

of kinase enzyme, might increase during this time due to some unknown factor that is somehow dependent on BldG. The fact that there is still unphosphorylated BldG present by 48 hours post-inoculation when sporulation begins, implies that the target sigma factor remains active in the later stages of development, an idea that is supported by the observed transcription pattern of *bldN*.⁷ Presumably, the level of ORF3 protein (which is likely controlled in part by protein degradation) is sufficiently low such that there is enough active BldG protein present to bind all the ORF3 protein and inhibit its activity.

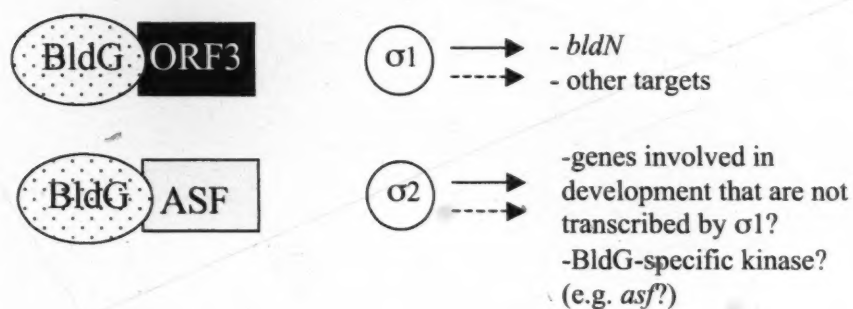
While the model described above accounts for many of the observations made, it is not clear based on this model why BldG phosphorylation (and therefore inactivation) would occur after the target sigma factor(s) are released (i.e. after 15 hours post-inoculation) if the sigma factor(s) remains active throughout aerial hyphae formation and sporulation. One would expect that phosphorylation of BldG might occur as a control mechanism to shut off transcription by the sigma factor(s) later in development, however in terms of *bldN* expression, this does not appear to be the case. Also, the above model assumes that BldG only interacts with ORF3 to regulate the activity of one or more sigma factors, however the observed excess of BldG protein produced during growth compared to ORF3 protein levels has led to the hypothesis that BldG may interact with ORF3 and with another anti-sigma factor-like protein in *S. coelicolor*. An alternate model (Figure 4.6.2) is therefore proposed which attempts to take these issues into account. As with the previous model, sigma factor inhibition is likely to occur during early vegetative growth, when BldG is presumably in the phosphorylated form. It is intriguing to speculate that phosphorylation of BldG in this case might be carried out by a second interacting anti-sigma factor (ASF) that possesses serine kinase activity towards BldG. Also, ORF3 and

Figure 4.6.2 Schematic diagram of a model proposing the regulation of sigma factors by BldG, ORF3 and by another anti-sigma factor-like protein (ASF). (A) During early vegetative growth (<15 hours post-inoculation), BldG (dotted oval) is likely phosphorylated and inactivated by a kinase [possibly the second interacting anti-sigma factor itself (ASF) itself]. ASF (gray box) and ORF3 (black box) might then bind to two different sigma factors (σ_1 , σ_2) and inhibit transcription by these sigma factors. (B) By the time late vegetative growth/transition phase occurs (15 hours post-inoculation), BldG is predominantly in the active, unphosphorylated form, possibly because of inefficient phosphorylation by the kinase, or because of the activation of a BldG-specific phosphatase enzyme. BldG may then bind to both ORF3 and ASF, allowing transcription of genes that may or may not be recognized by both sigma factors. The solid and dotted arrows indicate that the dependence of the target genes on the sigma factor(s) may be either direct or indirect. (C) Once pigmented antibiotic and aerial hyphae are both visible (24 hours post-inoculation), BldG is present in both the active and inactive forms. This might allow one anti-sigma factor (e.g. ASF) to bind to its cognate sigma factor (e.g. σ_2) and turn off transcription, while the other anti-sigma factor (e.g. ORF3) might still be inactivated by the remaining unphosphorylated BldG, thus allowing the second sigma factor (e.g. σ_1) to continue transcribing genes.

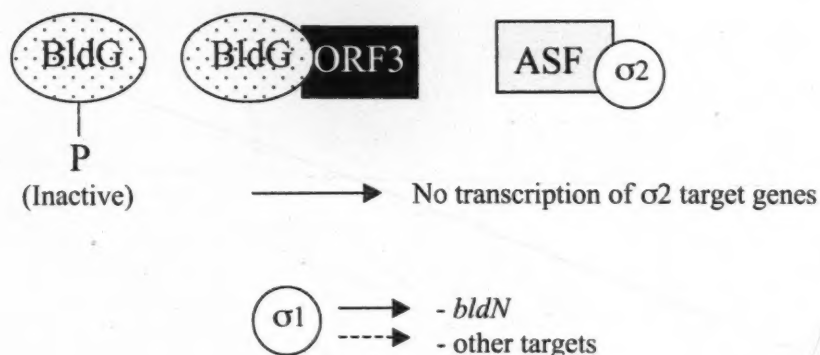
(a) Early vegetative growth (<15 hours post-inoculation):



(b) Late vegetative growth/transition phase
(15 hours post-inoculation):



(c) Onset of aerial hyphae formation and
antibiotic production (24 hours post-inoculation):



ASF might bind to different target sigma factors (designated $\sigma 1$ and $\sigma 2$) during early vegetative growth, which may or may not recognize similar promoter sequences. Once unphosphorylated BldG accumulates (due to either inefficient phosphorylation by ASF or by another kinase, or to the activity of a phosphatase), however, it may bind to both ORF3 and ASF, resulting in release of both target sigma factors which then proceed to transcribe genes involved in development. By the time pigmented antibiotics and aerial hyphae are both present, the levels of BldG and P-BldG are equivalent, with the increase in P-BldG due to either the BldG-dependent increase in expression of the kinase gene (e.g. that encoding ASF), or to the BldG-dependent increase in kinase activity, the mechanism of which is unknown. As such, there may be just enough BldG present to bind only one of the anti-sigma factor proteins (e.g. ORF3), which would allow one target sigma factor (e.g. $\sigma 1$) to continue to transcribe genes involved in development (e.g. *bldN*, or *bldN*-specific activator). On the other hand, the uninhibited anti-sigma factor protein (e.g. ASF) would be free to form a complex with the corresponding target sigma factor (e.g. $\sigma 2$), thereby shutting off transcription by that sigma factor.

It is obvious from both models proposed above that a number of unanswered questions remain. Most important, the nature of key proteins remains unknown, including the BldG-specific kinase and the sigma factor(s) regulated by BldG/ORF3. The isolation of a binding partner for BldG other than ORF3 might lead to the identification of the kinase enzyme (as suggested in the second model). Alternatively, the use of DNA microarrays might shed some light as to the nature of this protein, since the BldG-specific kinase was shown to be partially dependent on BldG itself. Such experiments might also allow for the identification of additional genes dependent on

ORF3 and/or BldG, which in turn might lead us to identify the target sigma factor(s), since the sigma factor responsible for transcription of one or more of these new targets might already be known. Alternatively, it may be possible to pull out the target sigma factor(s) from *S. coelicolor* crude extracts using purified ORF3 protein. It needs to be determined whether BldG, ORF3 (and maybe ASF) and the target sigma factor(s) are actually present in *S. coelicolor* during early vegetative growth (<15 hours post-inoculation) as the models assume, and whether BldG is present exclusively in the phosphorylated form at this time. Also, the phosphorylation of BldG needs to be further investigated, since it is not known what cellular conditions favor the phosphorylation of BldG (i.e. do ATP levels play a role as observed with RsbV?) or whether phosphorylation is controlled exclusively by the BldG-specific kinase itself, or whether a phosphatase enzyme is involved as observed in *Bacillus* and in other organisms. Such information is critical to the understanding of why phosphorylation of BldG might occur at different stages of development (i.e. during early vegetative growth, and then again as aerial hyphae and pigmented antibiotics become visible) and why only half of the BldG protein is phosphorylated in one case. A search of the *S. coelicolor* genome revealed at least 44 RsbP-related phosphatase enzymes, any of which might function to control the level of phosphorylated BldG in the organism. Alternatively, it is possible that one of the 16 RsbW-like proteins that are predicted to possess both kinase and phosphatase activity might control the reversible phosphorylation of BldG. Whether BldG and ORF3 interact *in vivo* to regulate a sigma factor as proposed also remains to be determined, as further examination of protein-protein interactions will need to be performed, either by chemical cross-linking using alternative cross-linking reagents, or by other protein binding

experiments. Despite all of these unanswered questions, however, this research has been successful in the characterization of *bldG* and of *orf3* to some extent, and the information obtained will hopefully contribute a piece to the larger puzzle of differentiation in *S. coelicolor*, so that one day we may all see the entire picture.

Chapter 5:

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